Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:2145-2157
DOI: 10.1159/000488068
Published online: March 12, 2018
Accepted: January 09, 2018

Original Paper

High-Concentrate Diet-Induced Change of Cellular Metabolism Leads to Decreases of Immunity and Imbalance of Cellular Activities in Rumen Epithelium

Zhongyan Lu[a] Hong Shen[b,c] Zanming Shen[a]

[a]Key Lab of Animal Physiology and Biochemistry, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu, [b]College of Life Science, Nanjing Agricultural University, Nanjing, Jiangsu, [c]Bioinformatics Center, Nanjing Agricultural University, Nanjing, Jiangsu, China

Key Words
Cellular metabolism • Epithelial immunity • Cellular activities • Rumen epithelium • RNA-seq • Gene regulatory network • Dietary concentrate

Abstract
Background/Aims: In animals, the immune and cellular processes of tissue largely depend on the status of local metabolism. However, in the rumen epithelium, how the cellular metabolism affects epithelial immunity, and cellular processes, when the diet is switched from energy-rich to energy-excess status, with regard to animal production and health, have not as yet been reported. ***Methods:*** RNA-seq was applied to compare the biological processes altered by an increase of dietary concentration from 10% to 35% with those altered by an increase of dietary concentration from 35% to 65% (dietary concentrate: the non-grass component in diet, including corn, soya bean meal and additive. High concentrate diet composed of 35% grass, 55% corn, 8% soya bean meal and 2% additive). In addition to the functional analysis of enriched genes in terms of metabolism, the immune system, and cellular process, the highly correlated genes to the enriched metabolism genes were identified, and the function and signaling pathways related to the differentially expressed neighbors were compared among the groups. ***Results:*** The variation trends of molar proportions of ruminal SCFAs and those of enriched pathways belonging to metabolism, immune system, and cellular process were altered with the change of diets. With regard to metabolism, lipid metabolism and amino acid metabolism were most affected. According to the correlation analysis, both innate and adaptive immune responses were promoted by the metabolism genes enriched under the 65% concentrate diet. However, the majority of immune responses were suppressed under the 35% concentrate diet. Moreover, the exclusive upregulation of cell growth and dysfunction of cellular transport and catabolism were induced by the metabolism genes enriched under the 65% concentrate diet. On the contrary, a balanced regulation of cellular processes was

Z. Lu and H. Shen contributed equally to this work.

Zanming Shen
Weigang No.1, Nanjing Agricultural University,
Nanjing (China)
Tel. 0086-25-8496493, Fax 0086-25-848936711 E-Mail zmshen@njau.edu.cn

detected under the 35% concentrate diet. ***Conclusions:*** These results indicated that the alterations of cellular metabolism promote the alterations in cellular immunity, repair, and homeostasis in the rumen epithelium, thereby leading to the switch of concentrate effects from positive to negative with regard to animal production and health.

Introduction

The rumen is the most important organ for the fermentation and absorption of dietary nutrients in ruminant animals. The fermentation products of the rumen, mainly short chain fatty acids (SCFAs), meet approximately 80% of the energy needs for these animals, and therefore, SCFAs play important roles in the maintenance of energy homeostasis of ruminants [1]. Concentrate is a type of rapidly fermentable substance in the diet and provides the majority of energy for the activities of rumen microbiota and rumen epithelium. Previous studies have shown that increase of dietary concentrate (from 10% to 35%, named energy-rich status) promotes rumen epithelial cell proliferation and enlarges the papillae size in young goats [2]. The increase of papillae size is a response that maximizes the epithelial surface area for absorption. Furthermore, the energy-rich diet facilitates the absorption of nutrients and ions [3], via stimulating the expression of their corresponding carriers in the rumen epithelium [4, 5], and maintaining the immune homeostasis in the rumen [6]. However, the further increase of dietary concentrate to 65%-85% (energy-excess status) causes the excessive ruminal SCFAs production and low ruminal pH. The latter two could induce parakeratosis, and accelerates sloughing and delamination in the stratified squamous of the rumen epithelium. These morphological changes would lead to the disruption of tight junctions between cells and increases permeability or paracellular transport for toxic substances from lumen to subepithelial tissue [7], thereby producing negative effects on animal production and on animal health [8]. Alterations of microbial metabolites and damage to the rumen epithelium have been reported to be important reasons for these negative effects on the animals. However, the way that the metabolic, immune, and cellular processes (including cell growth and death, cell community, transport and catabolism, and cell motility) alter in the rumen epithelium and their impacts on each other, when the diet is switched from energy-rich to energy-excess status, have not as yet been reported.

Metabolism is well known to fuel the activities of cells. Accordingly, the immune and cellular processes of tissue largely depend on the status of local metabolism. In ruminant animals, the metabolism of amino acids generates a wide range of effects on animal immunity and production [9]. Recent studies in monogastric animals have shown that lipid metabolism in tissue plays important roles in the transformation of immune cells (including macrophages, dendritic cells, and lymphocytes) between an anti-inflammatory state and a pro-inflammatory state, thereby contributing to the generation of either a pathogenic and inflammatory environment or a non-inflammatory and protective environment in the corresponding tissue [10-12]. Diet is an important factor affecting the metabolism status of tissues in animals. In ruminants, long-term high-concentrate diet suppresses their lipid metabolism, leading to a decrease in animal production [13]. Similarly, in the monogastric animals, long-term high-calorie diet results in the derangement of glucose and lipid metabolism that subsequently induces the dysfunction of the immune system and metabolism of the animals [14]. However, to date, the influence of cellar metabolism on the immune and cellular processes of rumen epithelium, under both energy-rich and energy-excess status, are unknown. We infer that, in the rumen epithelium, the change of cellar metabolism leads to changes of the immunity and cellular activities, when the diet is shifted from energy-rich to energy-excess status.

RNA-seq is a powerful technology that produces a complete view of the biological processes occurring in the investigated tissue. Moreover, by the combination of bioinformatics analysis, the gene regulatory network and functional interactions in the tissue can be clarified. In this study, we obtained RNA-seq data from the rumen epithelium of goats fed with 10%,

35%, and 65% dietary concentrate, respectively. In a comparison of metabolic processes, immune processes, and cellular processes altered by the shift of dietary concentrate from 10% to 35% and those altered by the shift of dietary concentrate from 35% to 65%, we identified the differences in the metabolic, immune, and cellular processes in the rumen epithelium between an energy-rich status and an energy-excess status. Subsequently, we used correlation analysis to identify the highly related genes (neighbors) to those genes involved in the altered metabolic processes, and then identified neighbors, referred to as candidate neighbors, that related to the immune processes and cellular processes according to the functional annotation. Finally, we dug out signaling pathways that linked metabolism-related genes and candidate neighbors on the correlation network. By this process, we aimed to clarify the impacts of differential metabolic processes between energy-rich and energy-excess status on the immune and cellular processes of the rumen epithelium. This study would benefit to develop new and potential technique, by using adequate dietary intervention, to modulate rumen epithelial growth and function.

Materials and Methods

Ethics approval

This study was 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) and the Care and Use of Animals (Nanjing Agricultural University, 1999).

Animals

Nine male goats (Boer × Yangtze River Delta White, aged 4 months) were randomly allocated into three groups and received a diet of 35% hay plus 65% concentrate (HC group, n= 3), a diet of 65% hay plus 35% concentrate (MC group, n= 3), and a diet of 90% hay plus 10% concentrate (LC group, n= 3) (for all online suppl. material, see www.karger.com/doi/10.1159/000488068, Table S1). All goats were fed with two equal portions of the designated diet at 0800 and 1700 daily for 28 days. Water was freely available to all goats during the experimental period. On day 29, the goats were killed at a local slaughterhouse.

Sample Collection

On day 29, all goats were slaughtered at 6 h after receiving the morning feed. Ruminal fluids (20 mL) were strained through a 4-layer cheesecloth and immediately subjected to pH measurement. 5% HgCl2 solution (1 mL) was added to the fluid samples, which were subsequently stored at −20°C for the determination of SCFA concentration. Rumen tissue from the ventral blind sac was quickly excised and washed by using ice-cold phosphate-buffered saline (PBS; pH 7.4). The epithelium was subsequently separated from the muscle layers and cut into 1-2 cm^2 pieces. Several pieces were immediately fixed in 4% paraformaldehyde (PFA) (Sigma, St. Louis, MO, USA) for histomorphometric microscopy analysis. The remaining pieces were stored at −80°C for later extraction of epithelial RNA.

The ruminal SCFA concentrations were determined by using a chromatograph (HP6890N, Agilent Technologies, Wilmington, DE) as described by Yang et al. [15].

Morphological Study

The ruminal tissue (1 cm^2) was rinsed in PBS and the papillae were cut and the density was counted (papillae number/cm^2). The length and width of the ruminal papilla were measured directly on paraformaldehyde fixed papillae by using a sliding caliper. Ten papillae per animal were prepared for optical microscopy and analyzed according to the description of Odongo et al. [16]. In brief, single PFA-fixed papilla were embedded in paraffin and sectioned at a thickness of 6 μm. Each section was stained with hematoxylin and eosin and then mounted on a slide for microscopic analysis. Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA) was used to observe the morphology of the epithelium in all samples.

Epithelial RNA Extraction and Sequencing

Total RNA was extracted from the ruminal epithelium by using the RNAeasy Mini Kit (Qiagen, Shanghai, China) according to the manufacturer's instructions. RNA was quantified by using the NanoDrop 1000 spectrophotometer, and its integrity was evaluated by using the RNA 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). High-quality RNA (RNA integrity number > 9.0) was processed by using NEB Next Ultra RNA Library Prep Kit (New England Biolabs, Beijing, China) following the manufacturer's instructions. All libraries were sequenced via paired-end chemistry (PE125) on an Illumina HiSeq2500 platform (Illumina, San Diego, CA, USA) at Biomarker Technologies, Beijing, China.

Transcriptome Assembling and Differentially Expressed Gene Identification

Low-quality reads (including more than 50% low-quality bases (< Q30) and more than 10% ambiguous bases (N)) were first removed by using PRINSEQ v0.20.4 [17]. The NCBI goat genome annotation release version 101 was used to construct the reference genome by using Bowtie v1.2.0 [18]. High-quality reads were mapped to the reference genome by using TopHat v2.1.0 [19] with standard parameters. Each SAM output file from the TopHat alignment was used in the Cuffdiff program of Cufflinks v2.2.1 [20] as input files to test for differential gene expression. In the Cuffdiff program, only mapped reads were used to estimate the gene expression level of each gene transcript, and the gene expression values were subsequently normalized to the reads per kilobase of exon model per million reads (RPKM). In this study, only genes with more than 1 RPKM in at least one group of the samples were considered to be expressed.

GO and KEGG Annotation and Enrichment

GO annotation of expressed genes was performed by using InterProScan 5 [21] with the default parameters. Subsequently, the KOBAS 3.0 web server [22] was used to annotate the genes against the KEGG database with the default parameters. After function annotation, R program AnnotationForge package [23] was used to construct the annotation library that was employed in the followed GO enrichment analysis. Then, the R program clusterProfiler package [24] was used to perform the GO and KEGG enrichment analysis for the differentially expressed genes between the groups. The sets of upregulated genes and downregulated genes were used in the enrichment analysis, separately. Finally, the R program ggplot2 package [25] was used to visualize the enrichment results.

Regulatory Network Construction

The co-expressed genes were first identified by computing the spearman correlation coefficient (SCC) between pairs of genes across groups by using the R program. Only expressed genes were used in the correlation analysis. A threshold for the SCC value larger than 0.8 and *p*-value less than 0.01 was used to identify significantly co-expressed genes. Accordingly, two gene co-expression networks were constructed based on the SCC of the expressed genes between the LC group and MC group (referred to as the energy-rich network) and the SCC of the expressed gene between the MC group and HC group (referred to as the energy-excess network). Secondly, the genes (neighbors) that were highly correlated to the enriched metabolism genes of the KEGG enrichment analysis were picked up from the network, and subsequently, the signaling pathways and functions of the differentially expressed neighbors were retrieved from KEGG databases. In this article, the differentially expressed neighbors that regulated the immune response and cellular processes are referred to as immunity-neighbors and cell-neighbors, respectively. Finally, the genes enriched in the metabolism terms of the KEGG enrichment analysis, namely the differentially expressed immunity-neighbors under the energy-rich/energy-excess status, the targeted immune responses and the signaling pathways that were used by the neighbors to regulate the immune responses were assembled and referred to as the energy-rich/energy-excess metabolism-immunity network. In the same way, the metabolism genes enriched in the KEGG enrichment analysis, the differentially expressed cell-neighbors under energy-rich/energy-excess status, the targeted cellular process, and the signaling pathways that were used by the neighbors to regulate the cellular process were assembled and referred to as the energy-rich/energy-excess metabolism-cell networks. All correlation networks were visualized by using cytoscape 3.4.0.

Availability of data and material

The RNA-seq data are available in the NCBI under BioProject PRJNA385353.

Results

Histomorphometric Analysis of the Rumen Epithelium

Compared with that in LC group, the length of the ruminal papillae in MC group increased by 1.62 times (3.59±0.24 vs. 1.37±0.07 mm, $p<0.05$) and the density increased by 34.19% (120.33±12.66 vs. 89.67±3.79 papillae/cm^2, $p<0.05$, see online suppl. material, Table S2).Compared with that in MC group, the length of the ruminal papillae in HC group decreased by 29.53% (2.53±0.18 vs. 3.59±0.24 mm, $p<0.05$) and the density decreased by 18% (98.67±13.50 vs. 120.33±12.66 papillae/cm^2, $p<0.05$, see online suppl. material, Table S2). The papillae width did not differ significantly among all the groups ($p > 0.05$, see online suppl. material, Table S2). Microscopic observation showed that total layers of the stratum corneum, and the stratum granulosum, and the stratum spinosum, and the stratum basale in rumen papillae of the MC group were thicker than those in LC and HC group (Fig. 1). However, in HC group, the number of leukocyte in the subepithelial tissue tended to be increased (Fig. 1).

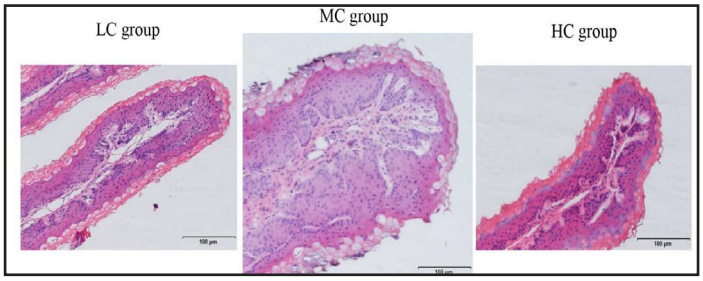

Fig. 1. Effects of LC, MC, and HC diet on the morphology of rumen epithelium.

Table 1. Effects of LC, MC and HC diet on the fermentation characteristics of goat rumen. [1]Values are mean ± standard error (SE). [2]MC group is set as control. $p < 0.05$ in the two-tailed T-test of LC group and MC group. [b]$p < 0.05$ in the two-tailed T-test of MC group and HC group

	LC	MC	HC
Acetate (mM)	54.56 ± 2.47	77.37 ± 4.98 [a]	117.02 ± 6.71 [b]
Acetate (%)	77.56 ± 0.69	75.83 ± 2.02[a]	83.02 ± 1.41[b]
Butyrate (mM)	10.84 ± 1.82	17.35 ± 1.38 [a]	16.01 ± 1.83
Butyrate (%)	15.41 ± 0.71	17.02 ± 1.39[a]	11.36 ± 1.21 [b]
Propionate (mM)	4.95 ± 0.68	7.29 ± 0.53 [a]	7.92 ± 0.41
Propionate (%)	7.04 ± 0.49	7.15 ± 2.02[a]	5.62 ± 0.42 [b]
Total SCFA (mM)	70.34 ± 4.96	101.96 ± 4.72[a]	140.95 ± 5.07 [b]
pH	6.70 ± 0.04	6.38 ± 0.03[a]	6.11 ± 0.04 [b]

Comparisons of Rumen pH and SCFAs Concentrations among the Groups

The concentration of the total SCFA (TSCFA), the pH, and the molar proportions of the major SCFAs (acetate, propionate, and butyrate) at 6 h after the morning feed ($p < 0.05$) are shown in Table 1.

Compared with the LC group, the molar proportions of butyrate and propionate and the concentration of TSCFA were significantly increased in the MC group, whereas the molar proportion of acetate and ruminal pH was significantly decreased.

Compared with the MC group, the molar proportion of acetate and the concentration of TSCFA were significantly increased in the HC group, whereas the molar proportions of butyrate and propionate and the ruminal pH were significantly decreased.

Expression Profiles of Genes in the Rumen Epithelium

RNA-seq generated a total of 2.9 G raw reads (average 320 M reads per sample, range 280–360 M) and 2.2 G clean reads (average 245 M reads per sample, range 214–292 M). On average, 93% of the clean reads were successfully mapped to the NCBI goat genome

Fig. 2. Variation trends of commonly enriched KEGG terms under the energy-rich diet and the energy-excess diet.

annotation release version 101. Finally, a total of 12, 400, 11, 833, and 11, 471 genes were detected as being expressed in the rumen epithelium of the LC group, MC group, and HC group, respectively.

Compared with the gene expression in the LC group, 403 genes were significantly upregulated ($\log_2$(MC/LC) >1, *q* value<0.05) and 521 genes were significantly downregulated ($\log_2$(MC/LC) <-1, *q* value<0.05) in the MC group. Compared with the gene expression in the MC group, 273 genes were significantly upregulated ($\log_2$(HC/MC) >1, *q* value<0.05) and 295 genes were significantly downregulated ($\log_2$(HC/MC) <-1, *q* value<0.05) in the HC group.

Gene Orthology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis

Enrichment analysis of differentially expressed genes between the LC and MC groups showed that 96 enriched GO terms (biological process, level 4) and 31 enriched KEGG pathways were upregulated in the MC group. Moreover, 140 enriched GO terms (biological process, level 4) and 57 enriched KEGG pathways were downregulated in the MC group. Enrichment analysis of differentially expressed genes between MC and HC groups showed that 110 enriched GO terms (biological process, level 4) and 25 enriched KEGG pathways were upregulated in the HC group. Furthermore, 75 enriched GO terms (biological process, level 4) and 17 enriched KEGG pathways were downregulated in the HC group.

A comparison of the GO terms enriched under the energy-rich status with those enriched under the energy-excess status revealed that a total of 63 GO terms were commonly enriched under both status (see online suppl. material, Fig. S1). However, 173 GO terms were exclusively enriched under the energy-rich status, and 122 GO terms were exclusively enriched under the energy-excess status. A comparison of the KEGG pathways enriched under the energy-rich status with those enriched under the energy-excess status showed that a total of 27 KEGG pathways were commonly enriched by both diets (Fig. 2). However, 63 KEGG pathways were exclusively enriched under the energy-rich status, and 17 KEGG pathways were exclusively enriched under the energy-excess status. Notably, the majority of the GO terms and KEGG pathways commonly enriched under both status showed the opposite trends (namely, upregulated under the energy-rich status, but downregulated under the energy-excess status; or vice versa).

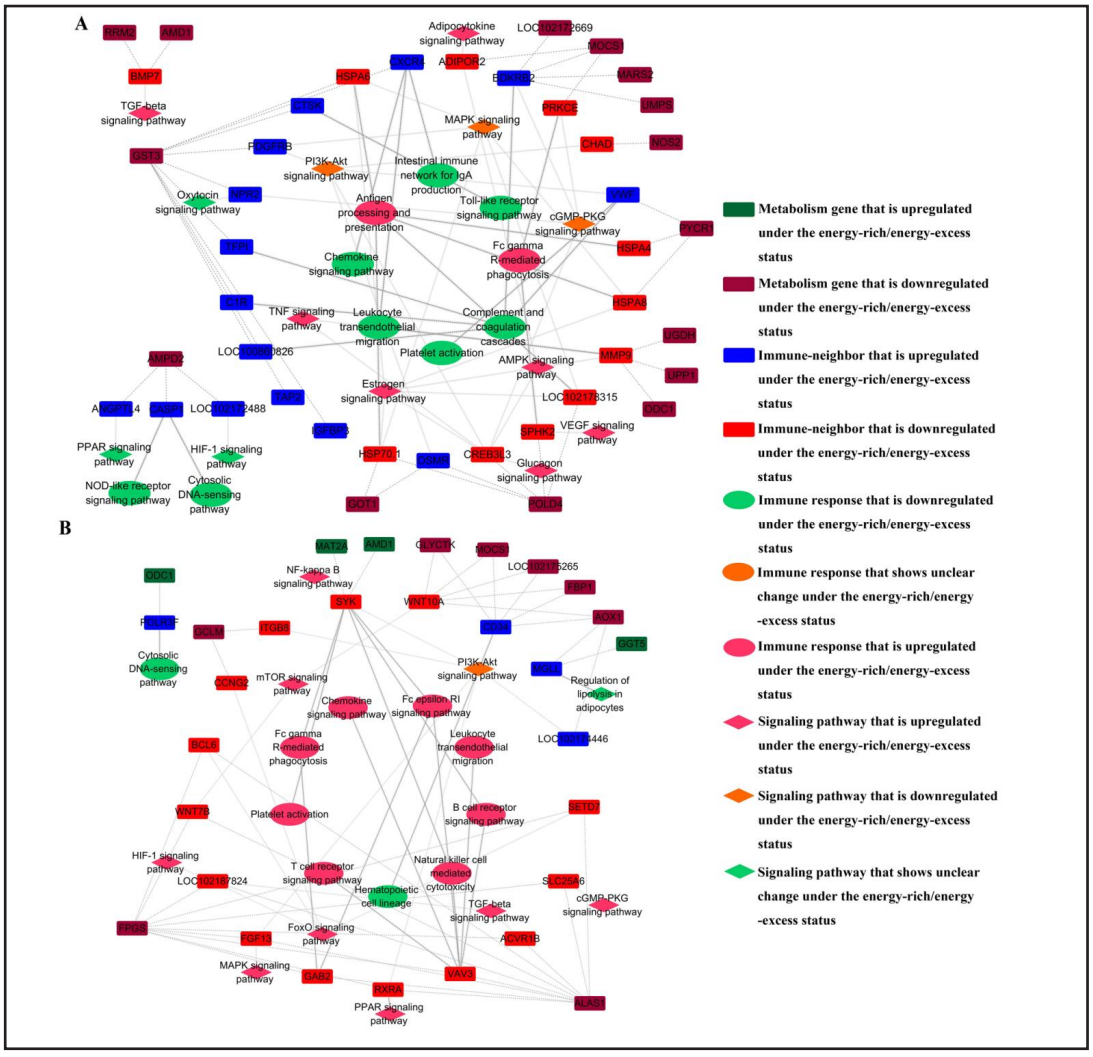

Fig. 3. Comparison of regulatory effects of differentially expressed genes, enriched in metabolism terms, on the immune system between the energy-rich feeding status and the energy-excess feeding status.

Function Annotation of Enriched Metabolism Genes following KEGG Enrichment Analysis

KEGG enrichment analysis of differentially expressed genes between the LC group and MC group showed that 68 metabolism genes were enriched, and the expression of these genes was significantly upregulated under the energy-rich status. At the same time, the KEGG enrichment analysis of differentially expressed genes between the MC group and HC group showed that 39 metabolism genes were enriched. Among them, the expression of 29 genes was significantly upregulated, whereas 10 genes were significantly downregulated under the energy-excess status. A comparison of the metabolism terms enriched under the energy-rich status and those enriched under the energy-excess status detected 4 terms (the nodes colored with light yellow (see online suppl. material) in Fig. S2) that were exclusively upregulated under the energy-rich status, whereas 14 terms (the nodes colored with light blue (see online suppl. material) in Fig. S2) were exclusively regulated under the energy-excess status. Additionally, arginine and proline metabolism, cysteine and methionine metabolism, and glutathione metabolism (the nodes colored with pink (see online suppl. material) in Fig. S2), which were upregulated under the energy-rich status, were downregulated under the energy-excess status.

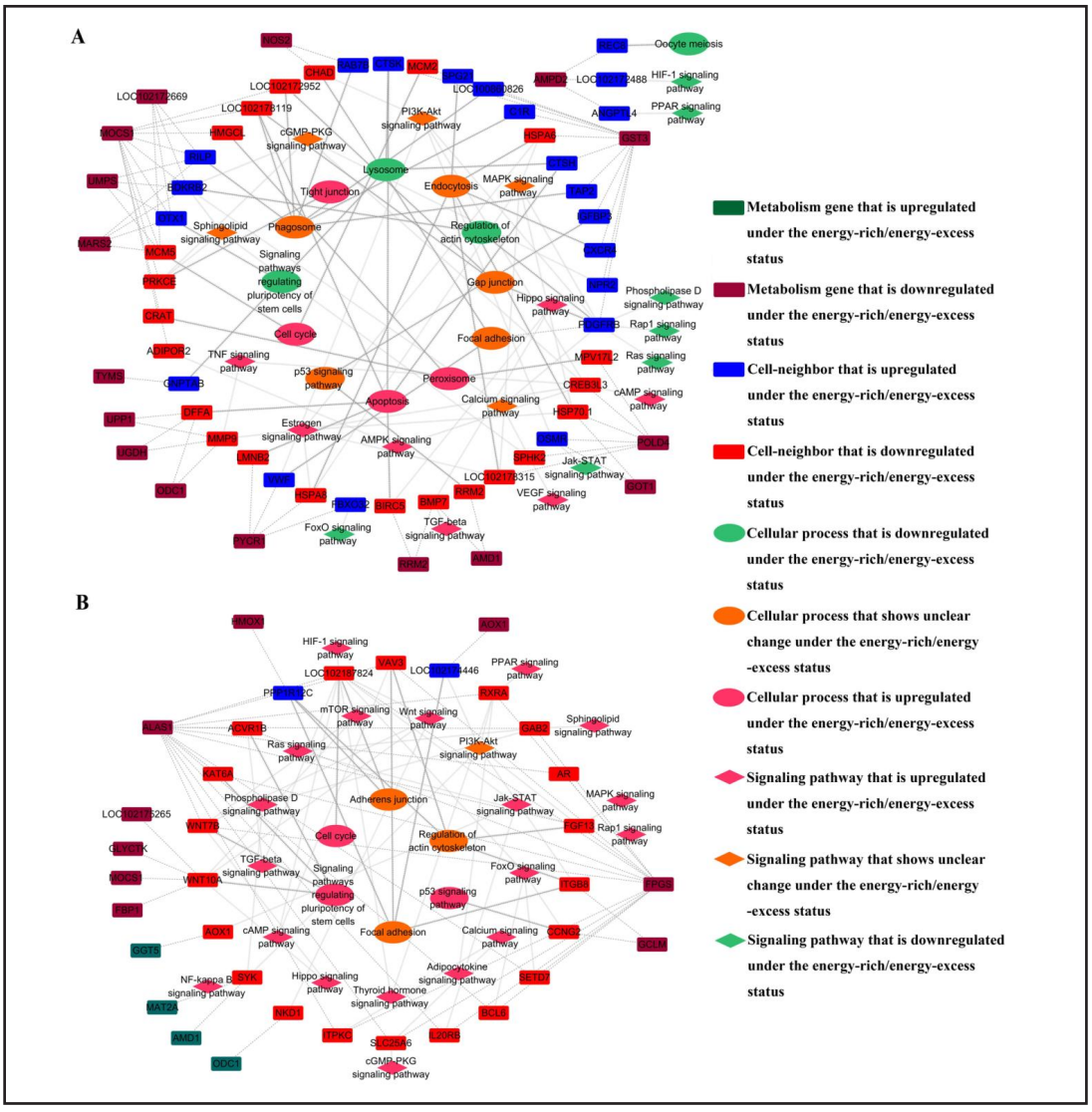

Fig. 4. Comparison of regulatory effects of differentially expressed genes, enriched in metabolism terms, on the cellular process between the energy-rich status and the energy-excess status.

Function Annotation of Enriched Immune System Genes following KEGG Enrichment Analysis

KEGG enrichment analysis of differentially expressed genes between the LC group and MC group showed that 89 immune system genes were enriched, and the expression of these genes was significantly downregulated under the energy-rich status. At the same time, KEGG enrichment analysis of differentially expressed genes between the MC group and HC group showed that 15 immune system genes were enriched. Among them, the expression of 8 genes was significantly upregulated, whereas 5 genes were significantly downregulated under the energy-excess status. A comparison of the immune responses enriched under the energy-rich status and those enriched under the energy-excess status revealed 5 responses (the oval nodes colored with light blue (see online suppl. material) in Fig. S3) and 6 signaling pathways that regulated the immune responses (the diamond nodes colored with orange (see online suppl. material) in Fig. S3) were exclusively downregulated under the energy-rich status, whereas one response (the oval nodes colored with orange (see online suppl. material) in Fig. S3) and one signaling pathway that regulated the immune responses (the diamond colored with brown (see online suppl. material) in Fig. S3) were exclusively

downregulated under the energy-excess status. Additionally, 4 responses (the nodes colored with violet (see online suppl. material) in Fig. S3) and 2 signaling pathways that regulate the immune responses (the diamond colored with pink (see online suppl. material) in Fig. S3) were downregulated under the energy-rich status, whereas all of them were upregulated under the energy-excess status.

Function Annotation of Enriched Cellular Process Genes following KEGG Enrichment Analysis

KEGG enrichment analysis of differentially expressed genes between the LC group and MC group showed that 105 genes were enriched in terms of cellular process. Among them, the expression of 48 genes was significantly upregulated, and the expression of 57 genes was significantly downregulated under the energy-rich status. At the same time, KEGG enrichment analysis of differentially expressed genes between the MC group and HC group showed that 24 cellular process genes were enriched. Among them, the expression of 5 genes was significantly upregulated, whereas 19 genes were significantly downregulated under the energy-excess status. In a comparison of the cellular processes enriched under the energy-rich status with those enriched under the energy-excess status, 6 processes (the nodes colored with pink (see online suppl. material) in Fig. S4) were exclusively upregulated under the energy-rich status, and 2 processes (the nodes colored with light green (see online suppl. material) in Fig. S4) were exclusively downregulated under the energy-rich status. Only the regulation of the actin cytoskeleton was exclusively downregulated under the energy-excess status. Additionally, signaling pathways regulating stem cell pluripotency, which was upregulated under the energy-rich status, was downregulated under the energy-excess status.

Regulatory Network of Metabolic Genes Enriched following KEGG Enrichment Analysis

First, 89 differentially expressed immunity-neighbors (compared with the LC group, 5 genes were significantly upregulated, and 84 genes were significantly downregulated in the MC group) were detected in the energy-rich network. On the other side, 13 differentially expressed immunity-neighbors (compared with the MC group, 9 genes were significantly upregulated, and 4 genes were significantly downregulated in the HC group) were detected in the energy-excess network. At the same time, 105 differentially expressed cell-neighbors (compared with the LC group, 48 genes were significantly upregulated, and 57 genes were significantly downregulated in the MC group) were detected in the energy-rich network, and 24 differentially expressed cell-neighbors (compared with the MC group, 5 genes were significantly upregulated, and 19 genes were significantly downregulated in the HC group) were detected in the energy-excess network.

With regard to the energy-rich metabolism-immunity network, 10 kinds of immune responses (oval nodes in Fig. 3A) and 15 signaling pathways that were related to the immune responses (diamond nodes in Fig. 3A) show the changes under the energy-rich status. Among these responses, 7 were downregulated (oval nodes colored with green in Fig. 3A), whereas one was upregulated (oval nodes colored with pink in Fig. 3A) under the energy-rich status. With regard to the energy-rich metabolism-immunity network, 10 kinds of immune responses (oval nodes in Fig. 3B) and 10 signaling pathways that were related to the immune responses (diamond nodes in Fig. 3B) show the changes under the energy-excess status. Among these responses, two were downregulated (oval nodes colored with green in Fig. 3B), whereas 8 were upregulated (oval nodes colored with pink in Fig. 3B) under the energy-rich status. In a comparison of the targeted responses between the energy-rich and energy-excess metabolism-immunity networks, 5 responses were changed under both status. Meantime, chemokine signaling pathway, leukocyte transendothelial migration, and platelet activation, which were all downregulated under the energy-rich status, were upregulated under the energy-excess status.

Finally, with regard to the energy-rich metabolism-cell network, 13 kinds of cellular processes (oval nodes in Fig. 4A) and 19 signaling pathways that regulated cellular process

(diamond nodes in Fig. 4A) show the changes under the energy-rich status. Among these processes, four were downregulated (oval nodes colored with green in Fig. 4A), whereas four were upregulated under the energy-rich status (oval nodes colored with pink in Fig. 4A). According to the energy-excess metabolism-cell network, 6 kinds of cellular processes (oval nodes in Fig. 4B) and 20 signaling pathways that regulated cellular process (diamond nodes in Fig. 4B) show the changes under the energy-excess status. Among these processes, except for three that showed unclear changes (the process was regulated by both of the upregulated genes and downregulated genes; oval nodes colored with orange in Fig. 4B), all the others were upregulated (oval nodes colored with pink in Fig. 4B) under the energy-excess status. In a comparison of the targeted processes between the energy-rich and energy-excess metabolism-cell networks, 5 processes were changed under both status. Among them, the cell cycle was upregulated under both status. However, the signaling pathway regulating the pluripotency of stem cells and the regulation of actin cytoskeleton, which were downregulated under the energy-rich status, were upregulated under the energy-excess status.

Discussion

Our results showed that shift the dietary level from energy-low to energy-rich status increased ruminal SCFAs concentration and reduced ruminal pH moderately, together with enlarged papillae size. However, further enhance of energy level to energy-excess status caused higher SCFAs concentration and acidic pH, eliminating the stimulating effect of energy-rich diet. SCFAs are known to promote the rumen papillae growth in neonatal sheep [26], and yang goats [2]. Butyrate stimulates cell proliferation and enlarges the papillae size [27]. Our study further indicates that ruminal pH may influence rumen papillae growth through synergistically with SCFAs.

To date, the effects of both energy-rich and energy-excess diets on the physiological processes that occur in the rumen epithelium have not as yet been reported. In this study, we have observed that, among the commonly enriched pathways in the metabolism terms, arginine and proline metabolism, cysteine and methionine metabolism and glutathione metabolism are upregulated under the energy-rich status, whereas they are downregulated under the energy-excess status.

We have studied the influences of epithelial metabolism on cell process. Proline metabolism regulates cell proliferation, apoptosis, and autophagy [28]. The functional study with KEGG enrichment analysis has further shown that, in the terms of cellular process, cell growth and death are upregulated, whereas endocytosis and phagosomes are downregulated under the energy-rich status. These results, together with the morphological results, indicate that the energy-rich diet promotes cell refreshing and damaged cell repair in the rumen epithelium. Concomitantly, under the energy-rich status, the tight junctions and gap junctions of the epithelial cells are strengthened indicating the consolidation of the epithelial barrier. However, none of the mentioned cellular processes is affected by the energy-excess status, indicating the disappearance of the positive effects on the tissue repair and epithelium barrier when dietary concentrate goes beyond 65%. In our study of the regulatory effects of metabolism-related genes on the cellular process, we determined that transport and catabolism (including lysosome, peroxisome, phagosome, and endocytosis) and cell death (cell apoptosis) were exclusively regulated under the energy-rich status. Endocytosis is a mechanism for cells to remove ligands, including microbial pattern recognition receptors (MPRRs), from the cell surface, thereby contributing to the suppression of the immune response to the nonpathogenic bacteria in the rumen. Peroxisomes are essential organelles of the cells, which play an important role in lipid homeostasis and free radical detoxification. The phagosomes and lysosomes help the cells to kill and degrade the invading bacteria. Cell apoptosis helps the organism to clear the damaged cells, thereby contributing to tissue repair and barrier integrity. Accordingly, these processes, regulated under the energy-rich status, play important roles in the maintenance of function

and metabolic homeostasis in the rumen epithelium. Secondly, different from energy-excess status, the regulation of cell growth and death (including cell cycle and apoptosis) and of cell community (including tight junction and signaling pathways regulating the pluripotency of stem cells) was bidirectional under the energy-rich status. Such kinds of regulation benefit the maintenance of homeostasis in the rumen epithelium. Taken together, our results indicate that the imbalance of cell growth and death and the dysfunction of transport and catabolism in the epithelial cells lead to the destruction of epithelial integrity under the energy-excess status.

Previous studies have shown the effects of metabolism on immune activity. Arginine metabolism plays an important role in host defense and tissue repair [29]. Cysteine is the major component of glutathione, whereas glutathione metabolism is reported to be beneficial to cell health and functions both by acting as an oxidant scavenger and by opposing the pro-inflammatory influence of hydrogen peroxide on cell signaling [30]. Furthermore, lipid metabolism is exclusively upregulated under the energy-rich status. Increases of free fatty acids, the products of lipolysis, in the tissue has been reported to induce the secretion of pro-inflammatory factors from both immune cells and adipose cells [14]. Our results suggest that, in the terms of the immune system, the responses of both innate immune cells and adaptive immune cells are suppressed under the energy-rich status. This is in agreement with our previous studies showing that an energy-rich diet decreases the expression of pro-inflammatory cytokines in the rumen epithelium, thereby contributing to the epithelial tolerance to the expanded commensal bacteria in the rumen [6]. However, under the energy-excess status, such suppression on the immune responses does not occur. Contrarily, platelet activation, leukocyte transendothelial migration, complement and coagulation cascades, and Fc epsilon RI signaling pathway that leads to the release of preformed granules from mast cells are promoted under the energy-excess status. Taken together, the metabolism status constructed under the energy-rich status is associated with the promotion of immune tolerance and the strengthening of defense and repair in the rumen epithelium. On the contrary, the metabolism status, especially the lipid metabolism and amino acids metabolism status, constructed under the energy-excess status is associated with the activation of immune responses and the suppression of cell functions in the rumen epithelium.

In our study of the regulatory effects of metabolism-related genes on the immune system, we have found that in epithelial and subepithelial tissues, under the energy-rich status, the immunity-neighbors suppress both of the innate immune responses (including the NOD-like receptor signaling pathway, Toll-like receptor signaling pathway, chemokine signaling pathway, complement and coagulation cascades, and cytosolic DNA-sensing pathway) and adaptive immune responses (including intestinal immune network for IgA production, leukocyte transendothelial migration, and platelet activation). On the contrary, under energy-excess status, the immunity-neighbors upregulate both the innate immune responses (including Fc gamma R-mediated phagocytosis, chemokine signaling pathway, and natural killer cell-mediated cytotoxicity) and the adaptive immune responses (leukocyte transendothelial migration, platelet activation, T cell receptor signaling pathway, and B cell receptor signaling pathway). In addition, the rapamycin (mTOR) signaling pathway, peroxisome proliferator-activated receptor (PPAR) signaling pathway, and ras-mitogen-activated protein kinase (MAPK) signaling pathways are exclusively upregulated under the energy-excess status. mTOR is an atypical serine/threonine kinase involved in regulating major cellular functions including growth and proliferation. Previous studies have demonstrated that the upregulation of the mTOR signaling pathway is one of the most commonly observed pathological alterations in the cancer cells [31]. Moreover, the upregulation of the mTOR-PPAR signaling pathway promotes lipid accumulation in the liver and therefore contributes to the obesity-induced dysfunction of the immune system and metabolism [32]. Then, the upregulation of the MAPK signaling pathway exaggerates the growth effects of insulin on the cells and promotes the autophagy and apoptosis processes of the cells [33]. Therefore, the upregulation of this signaling pathway might contribute to the pathological alterations of epithelial morphology under energy-excess status. Taken

together, the upregulation of these signaling pathways might exert the important roles in the switch of diet from energy-rich to energy-excess status.

Our data indicate that the increase in dietary concentration from the energy-rich pattern to the energy-excess pattern induces changes of cellular metabolism in the rumen epithelium via its effects on the molar proportions of ruminal SCFA. Subsequently, under the energy-excess feeding status, the altered cellular metabolism, especially the lipid metabolism and amino acid metabolism, declines the immunity and cellular repair in the rumen epithelium and, contrarily, promotes the excessive growth of epithelial cells, thereby leading to the loss of epithelial homeostasis and the damage of its integrity.

This is the first ever study to conduct a complete comparison of the biological processes altered under the energy-rich status and those altered under the energy-excess status in the rumen epithelium through RNA-seq method. Moreover, it is also the first ever study concerning the effects of cellular metabolism on the immune responses and cellular process in the rumen epithelium. Our results provide a better understanding of the negative/positive effects of dietary concentration on animal production and animal health. Moreover, it deepens our knowledge concerning the roles of cellular metabolism in the rumen physiology. This study provides clue for future rumen research that in addition to rumen fermentation, the role of epithelial metabolism on papillae morphology and immune activity should be considered, when the feeding strategy is used to modulate epithelial growth and function.

Acknowledgements

This work was supported by the Project Grant Natural Science Foundation of Jiangsu Province (BK20150654), the Chinese National "973" Project (2011CB100801), the Independent Innovation Project of Nanjing Agriculture University (KYZZ01628), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Disclosure Statement

No conflict of interests exists.

References

1 Bergman EN: Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 1990;70:567-590.
2 Gui H, Shen Z: Concentrate diet modulation of ruminal genes involved in cell proliferation and apoptosis is related to combined effects of short-chain fatty acid and pH in rumen of goats. J Dairy Sci 2016;99:6627-6638.
3 Etschmann B, Suplie A, Martens H: Change of ruminal sodium transport in sheep during dietary adaptation. Archives of animal nutrition 2009;63:26-38.
4 Metzler-Zebeli BU, Hollmann M, Sabitzer S, Podstatzky-Lichtenstein L, Klein D, Zebeli Q: Epithelial response to high-grain diets involves alteration in nutrient transporters and Na+/K+-ATPase mRNA expression in rumen and colon of goats. J Anim Sci 2013;91:4256-4266.
5 Yan L, Zhang B, Shen Z: Dietary modulation of the expression of genes involved in short-chain fatty acid absorption in the rumen epithelium is related to short-chain fatty acid concentration and pH in the rumen of goats. J Dairy Sci 2014;97:5668-5675.
6 Shen H, Chen Z, Shen Z, Lu Z: Maintaining stability of the rumen ecosystem is associated with changes of microbial composition and epithelial TLR signaling. MicrobiologyOpen 2017:10.1002/mbo1003.1436.
7 Steele MA, Croom J, Kahler M, AlZahal O, Hook SE, Plaizier K, McBride BW: Bovine rumen epithelium undergoes rapid structural adaptations during grain-induced subacute ruminal acidosis. Am J Physiol Regul Integr Comp Physiol 2011;300:R1515-1523.
8 Zebeli Q, Metzler-Zebeli BU: Interplay between rumen digestive disorders and diet-induced inflammation in dairy cattle. Res Vet Sci 2012;93:1099-1108.

9 Lobley GE: Control of the metabolic fate of amino acids in ruminants: a review. J Anim Sci 1992;70:3264-3275.
10 Newton R, Priyadharshini B, Turka LA: Immunometabolism of regulatory T cells. Nat Immunol 2016;17:618-625.
11 Schipper HS, Prakken B, Kalkhoven E, Boes M: Adipose tissue-resident immune cells: key players in immunometabolism. Trends Endocrinol Metab 2012;23:407-415.
12 Van den Bossche J, O'Neill LA, Menon D: Macrophage Immunometabolism: Where Are We (Going)? Trends Immunol 2017
13 Plaizier JC, Krause DO, Gozho GN, McBride BW: Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. Vet J 2008;176:21-31.
14 Ouchi N, Parker JL, Lugus JJ, Walsh K: Adipokines in inflammation and metabolic disease. Nat Rev Immunol 2011;11:85-97.
15 Yang W, Shen Z, Martens H: An energy-rich diet enhances expression of Na(+)/H(+) exchanger isoform 1 and 3 messenger RNA in rumen epithelium of goat. J Anim Sci 2012;90:307-317.
16 Odongo NE, Alzahal O, Lindinger MI, Duffield TF, Valdes EV, Terrell SP, McBride BW: Effects of mild heat stress and grain challenge on acid-base balance and rumen tissue histology in lambs. J Anim Sci 2006;84:447-455.
17 Schmieder R, Edwards R: Quality control and preprocessing of metagenomic datasets. Bioinformatics 2011;27:863-864.
18 Langmead B, Trapnell C, Pop M, Salzberg SL: Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009;10:R25.
19 Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL: TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 2013;14:R36.
20 Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L: Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 2010;28:511-515.
21 Mulder N, Apweiler R: InterPro and InterProScan: tools for protein sequence classification and comparison. Methods Mol Biol 2007;396:59-70.
22 Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, Kong L, Gao G, Li CY, Wei L: KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res 2011;39:W316-322.
23 Carlson M, Pages H: AnnotationForge: Code for Building Annotation Database Packages. 2017
24 Yu G, Wang LG, Han Y, He QY: clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 2012;16:284-287.
25 Wickham H: ggplot2: Elegant Graphics for Data Analysis., Springer-Verlag New York, 2009.
26 Lane MA, Jesse BW: Effect of volatile fatty acid infusion on development of the rumen epithelium in neonatal sheep. J Dairy Sci 1997;80:740-746.
27 Malhi M, Gui H, Yao L, Aschenbach JR, Gabel G, Shen Z: Increased papillae growth and enhanced short-chain fatty acid absorption in the rumen of goats are associated with transient increases in cyclin D1 expression after ruminal butyrate infusion. J Dairy Sci 2013;96:7603-7616.
28 Zareba I, Palka J: Prolidase-proline dehydrogenase/proline oxidase-collagen biosynthesis axis as a potential interface of apoptosis/autophagy. BioFactors 2016;42:341-348.
29 Gogoi M, Datey A, Wilson KT, Chakravortty D: Dual role of arginine metabolism in establishing pathogenesis. Curr Opin Microbiol 2016;29:43-48.
30 McCarty MF, DiNicolantonio JJ: An increased need for dietary cysteine in support of glutathione synthesis may underlie the increased risk for mortality associated with low protein intake in the elderly. Age (Dordr) 2015;37:96.
31 Xu K, Liu P, Wei W: mTOR signaling in tumorigenesis. Biochim Biophys Acta 2014;1846:638-654.
32 Li Z, Xu G, Qin Y, Zhang C, Tang H, Yin Y, Xiang X, Li Y, Zhao J, Mulholland M, Zhang W: Ghrelin promotes hepatic lipogenesis by activation of mTOR-PPARgamma signaling pathway. Proc Natl Acad Sci U S A 2014;111:13163-13168.
33 Zhong W, Zhu H, Sheng F, Tian Y, Zhou J, Chen Y, Li S, Lin J: Activation of the MAPK11/12/13/14 (p38 MAPK) pathway regulates the transcription of autophagy genes in response to oxidative stress induced by a novel copper complex in HeLa cells. Autophagy 2014;10:1285-1300.