Effects of High-Grain Diet with Buffering Agent on the Milk Protein Synthesis in Lactating Goats

Meilin He[1], Xintian Nie[2], Huanhuan Wang[1], Shuping Yan[1], Yuanshu Zhang[1]*

From the [1] The Key Laboratory of Animal Physiology and Biochemistry, Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, People's Republic of China.

[2] College of Engineering, Nanjing Agricultural University, Nanjing 210095, People's Republic of China.

Running Title: **Effect of buffering agent on milk protein synthesis**

*To whom correspondence should be addressed: Yuanshu Zhang: The Key Laboratory of Animal Physiology and Biochemistry, Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, People's Republic of China. zhangyuanshu@njau.edu.cn

Abstract

Feeding of straw as main roughage with numerous high-grain diets improves the performance of ruminants but it can easily lead to subacute ruminal acidosis. In recent years, buffering agent is applied to prevent the acid poisoning of ruminants and improve the production performance of ruminants in animal husbandry. it is necessary to understand feeding high-grain diet with buffering agent which transport carriers amino acids mainly take amino acids into the mammary gland and the signal mechanism of amino acids in the mammary gland synthesize milk proteins. To gain insight on the effects of a high-grain diet with buffering agent on the amino acids in the jugular blood, and the effects of amino acids on the synthesis of milk protein, commercial kit and high performance liquid chromatography (HPLC) were applied to determine the concentration of amino acids of jugular blood samples, quantitative real-time PCR, comparative proteomic approach and western blot were employed to investigate proteins differentially expressed in mammary tissues and the mechanism of amino acids on the synthesis of milk protein in mammary gland of lactating dairy goats fed high-grain diet with buffering agent or only high-grain diet.

Results showed that feeding high-grain diet with buffering agent to lactating dairy goats could outstanding increase amino acid content of jugular blood ($p<0.05$), and mRNA transcriptional level of amino acid transporters in the mammary gland were also increased; the CSN2 and LF protein expression level were significant higher by 2-DE technique，MALDI-TOF/TOF proteomics analyzer and western blot analysis further validated in mammary of lactating dairy goats compared with high-grain group; the

research on the mechanism of milk protein synthesis increasing suggested that it was related to the activation of mTOR pathway signaling.

Feeding of high-grain diet with buffering agent promoted the jugular vein blood of amino acids concentration, and more amino acids flowed into the mammary. In addition, milk protein synthesis was increased and the increase of milk protein synthesis was related to the activation of mTOR pathway signalling.

Keywords: buffering agent; lactating dairy goats; HPLC; comparative proteomic; amino acids; milk protein

Background

Due to reduction of per capita arable land, the degradation of grasslands, and shortage of green fodder resources and poor quality, the current feeding practices in the dairy industry improving feed quality by feeding high-grain diets. However, many studies have confirmed that feeding of straw as main roughage with numerous high-grain diets improves the performance of ruminants but it can easily lead to subacute ruminal acidosis (SARA) (1,2). SARA has observed a decline in ruminal pH below 5.6 fed such ration for 3-5 h (3).

Buffering agent is a chemical that enhances the acid-base buffer capacity of a solution. In recent years, it is applied to prevent the acid poisoning of ruminants and improve the production performance of ruminants in animal husbandry. A comprehensive study of three American universities has shown that adding 1.5% sodium bicarbonate and 0.8% magnesium oxide in the diets of early and mid-term lactating dairy cows can cause milk production and milk fat levels to increase significantly (4).

Kurokawa et al. added 80g of sodium bicarbonate every day when feeding the lactating dairy cows with salt which was less than 20% of standard, compared with the control group, the milk yield was 5.1% and the butterfat percentage was 0.15% below the standard, and compared with the control group, the standard milk was the increased by 5.1% and the rate of butterfat increased by 0.15% (5). In order to maintain ruminal pH in lactating dairy goats, buffering agent was added to the high-grain diets.

Milk protein is one of the determinants of milk quality, and milk proteins in the lactating ruminant mammary gland are primarily synthesized from circulating plasma amino acids (6). Previous studies have shown that amino acids can be taken into the mammary gland by the mammary epithelial cells through the transport carriers (7−9). Therefore, it is necessary to understand feeding high-grain diet with buffering agent

which transport carriers amino acids mainly take amino acids into the mammary gland and the signal mechanism of amino acids in the mammary gland synthesize milk proteins. In this way, it can alleviate SARA's symptoms and improve the milk protein production and lay a foundation for future research.

Methods

Experimental animals

A total of twelve healthy multiparous mid-lactating goats (body weight, 38 ± 8 kg, mean ± SEM, 3-5 weeks post-partum) at the age of 2-3 years were used in experiments. They were housed in individual stalls in a standard animal feeding house at Nanjing Agricultural University (Nanjing, China). Goats were randomly divided into two groups: high-grain group (HG, concentrate: forage = 60:40) and buffering agent group (BG, concentrate: forage = 60:40 with 10g $C_4H_7NaO_2$ and 10g $NaHCO_3$), six in each group. Dietary $C_4H_7NaO_2$ and $NaHCO_3$ were obtained from Nanjing Jian cheng Bioengineering Institute, China. The ingredients and nutritional composition of the diets are presented in Table 1. The goats were fitted with a rumen fistula and hepatic catheters two weeks before the experiment and were ensured that they recovered from the surgery. Animals were monitored for 2 weeks after surgery. Sterilized heparin saline (500 IU/ml, 0.3 ml/time) was administered at 8-hour intervals every day until the end of the experiment to prevent catheters from becoming blocked. During the experimental period of 20 weeks, goats were fed two times daily at 8.00 and 18.00, had free access to fresh water, and the feed amount met or exceeded the animal's nutritional requirements. The Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, People's Republic of China) approved all of the procedures (surgical procedures and care of goats).

Analysis of total amino acids

At the 20^{th} week, blood samples were collected from the jugular blood in 10 mL vacuum tubes containing sodium heparin. Blood was centrifuged at 3,000 × g for 15 min to separate plasma. The total amino acids concentration were determined by a Total Amino Acid assay kit (catalog no. A026, Jiancheng, Nanjing, China). The procedures were performed according to the manufacturer's instructions.

Analyses of amino acids profile by HPLC

Free amino acids of jugular blood samples were determined by high performance liquid chromatography (HPLC), it was performed as previously described by Shen et al (10). The HPLC system consisted of: Agilent1100 high-performance liquid chromatograph

system (Agilent Technologies, Waldbronn, Germany); scanning fluorescence detector (excitation 340 nm, emission 450 nm); chromatographic column (XTerra®MS C18, 4.6mm × 250mm, 5μm), which was purchased from waters (Waters Co., Milford, MA, USA). 20 kinds of standard amino acids (Aldrich chemical company) were given by professor Holey, and the purity of these amino acids were greater than 98%. The three-dimensional flow phase (solution A, methanol; solution B, acetonitrile; solution C, 10mmol/L phosphate buffer containing 0.3% tetrahydrofuran) was adopted. The gradient program was referred to Table 2, and oven temperature was 40 °C，the injection volume was 20 μL. The plasma samples were mixing with acetonitrile by 1:2(v/v), and were placed at 4°C for 30 min, then they were centrifuged at 12000 rpm for 30 min, and the supernatant fluids were collected for AA analysis. High pressure liquid chromatography analysis was performed after automatic pre-column derivatization with O-phthaldialdehyde (OPA) (11).

Quantitative Real-Time PCR (qRT-PCR)

After 20 weeks, goats were slaughtered after overnight fasting. All goats were killed with neck vein injections of xylazine (0.5 mg (kg body weight)$^{-1}$; Xylosol; Ogris Pharme, Wels, Austria) and pentobarbital (50 mg (kg body weight)$^{-1}$; Release; WDT, Garbsen, Germany). After slaughter, mammary tissue was collected and washed twice with cold physiological saline (0.9% NaCl) to remove blood and other contaminants. Mammary tissue samples were used for RNA and protein extraction. Total RNA was extracted from each mammary tissue sample using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's specifications and then reverse-transcribed into cDNA using commercial kits (Vazyme, Nanjing, China). All PCR primers were synthesized by Generay Company (Shanghai, China), and the primer sequences are listed in Table 3. PCR was performed using the AceQ qPCR SYBR Green Master Mix kit (Vazyme, Nanjing, China) and the MyiQ2 Real-time PCR system (Bio-Rad, USA) with the following cycling conditions: 95°C for 2 min, 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Glyeraldehyde 3-phosphate dehydrogenase (GAPDH) served as reference for normalization.

Protein extraction from mammary tissues

Protein extraction from mammary tissues was performed according the method described by Duanmu et al (12). The mammary tissue samples of all goats in BG in equal quality were mixed and washed three times with ice-cold saline containing 1 mM

PMSF, then homogenized in the ice-cold lysis buffer (2M thiourea, 7M urea, 50mM DTT, 2% (w/v) CHAPS, 0.5% (v/v) Bio-Lyte Ampholyte and 1mM PMSF) by 1:5 (w/v). The homogenates were kept at room temperature for 30 min, followed by centrifugation at 15,000 g for 30 min at 4 °C. The HG samples were treated in the same way. The samples were stored at -80 °C until analysis. The protein concentration of the supernatant was determined by RC DCTM (Bio-Rad, USA) kit.

Two-dimensional gel electrophoresis (2-DE)

The operation method of 2-DE was referred to our laboratory previous research by Jiang et al (13). The first dimension used was isoelectric focusing (IEF). The extracted protein (1000 mg) was loaded onto the 17 cm IPG gel strips (nonlinear, pH 3.0-10.0, Bio-Rad, USA) according to Chen et al (14). Passive rehydration (13 h with 50 V). IEF was performed with a voltage gradient of 250 V for 1 h, 500 V for 1 h, 2000 V for 1 h, 8000 V for 3 h, followed by holding at 8000 V until a total of at least 60 000 V-h was reached. Then IPG strips were equilibrated by serial incubation for 15 min in equilibration buffer (6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris-HCl (pH 8.8) and 1% (w/v) DTT) and in equilibration buffer containing 2.5% (w/v) iodoacetamide instead of 1% DTT. Equilibrated IPG strips were transferred onto the 12.5% SDS-PAGE for the second dimension (15). Gels were fixed in 12% trichloroacetic acid for 2 h, then stained with 0.08% (w/v) Coomassie Brilliant Blue G250 staining solution for 20 h. The excess of dye was removed with MilliQ water, and scanned with Molecular Imager (Versa Doc3000, Bio-Rad, USA). Standardization, background elimination, spot detection, gel matching and interclass analysis were performed as previously described using the PDQuest 8.0 software (Bio-Rad) (16). Three replicates were performed per sample. Protein spots were considered to be differentially expressed only if they showed 1.5-fold change in intensity, and satisfied the non-parametric Wilcoxon test ($p< 0.05$). Only the spots with the same changing trend in all three gels were considered for further analysis.

Trypsin digestion and MS analysis

Selected gel spots were manually excised and washed twice with MilliQ water. Trypsin digestion test was performed as described by Ura et al (17). The digested proteins were air-dried and analyzed by using a 4800 MALDI-TOF/TOF proteomics analyzer (Applied Biosystems, USA). A protein spot digested with trypsin was used to calibrate the mass spectrometer. A mass range of 800-3500 Da was used. Combined search (MS plus MS/MS) was performed using GPS Explorer TM software v3.6 (Applied

Biosystems, USA) and the MASCOT search engine (Matrix Science Ltd., UK). Proteins were considered as positive hints if at least two independent peptides were identified with medium (95%) or high (99%) confidence.

Western blot analysis

Protein was extracted from mammary tissue samples using lysis buffer (Cell Signaling) plus PMSF (1 mM) and total protein was quantified by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). The protein of all samples in BG in equal quality were mixed, the HG samples were treated in the same way. Four replicates were performed per sample. We isolated 30 μg of protein from each sample, which was subjected to electrophoresis on SDS-PAGE. The separated proteins were transferred onto nitrocellulose membranes (Bio Trace, Pall Co., USA). The blots were incubated with the following Cell Signaling Technology primary antibodies for overnight at 4^{o}C with a dilution of 1:1000 in block: rb-anti-Mammalian target of rapamycin (rb-anti-mTOR, #2983S), rb-anti-phospho-mTOR (rb-anti-p-mTOR, #5536S), rb-anti-P70 ribosomal protein S6 kinase (rb-anti-P70S6K, #9202S), rb-anti-phospho-P70S6K (rb-anti-p-P70S6K, #5536S), rb-anti-eukaryotic translation initiation factor 4E (rb-anti-eIF4E, #9742S), rb-anti-phospho-eIF4E (rb-anti-p-eIF4E, #9741S), rb-anti-eukaryotic elongation factor-2 kinase (rb-anti-eEF2K, #3692S), rb-anti-phospho- eukaryotic elongation factor-2 kinase (rb-anti-p-eEF2K, #3691S), rb-anti-eukaryotic elongation factor-2 kinase (rb-anti-eEF2, #2332S). The blot was incubated with ABclonal Technology primary antibody for overnight at 4^{o}C with a dilution of 1:1000 in block: rb-anti-beta-casein protein (rb-anti-CSN2, #A12749S). The blot was incubated with primary antibody for overnight at 4^{o}C with a dilution of 1:400 in block: rb-anti-lactoferrin (rb-anti-Lf, our laboratory) (18). A rb-anti-GAPDH primary antibody (a531, Bioworld, China, 1: 10,000) was also incubated with the blots to provide a reference for normalization. After washing the membranes, an incubation with HRP-conjugated secondary antibody was performed for 2 h at room temperature. Finally, the blots were washed and signal was detected by enhanced chemiluminescence (ECL) using the LumiGlo substrate (Super Signal West Pico Trial Kit, Pierce, USA). ECL signal was recorded using an imaging system (Bio-Rad, USA) and analyzed with Quantity One software (Bio-Rad, USA). The phosphorylation level of mTOR, P70S6K, eIF4E and eEF2K was determined by the ratio of p- mTOR to total mTOR, p-P70S6K to total P70S6K， p-eIF4E to total eIF4E and p-eEF2K to total eEF2K, respectively. The

expression level of eEF2, CSN2 and LF were determined by the ratio of eEF2 to GAPDH, the ratio of CSN2 to GAPDH and the ratio of LF to GAPDH.

Statistical analyses

Data were analyzed using the Statistical Package for Social Science (SPSS Inc., Chicago, IL, USA) and all data are presented as the mean ± SEM. The $2^{-\Delta\Delta Ct}$ method was applied to analyze the real-time PCR. Data were considered statistically significant if $P < 0.05$. The numbers of replicates used for statistics are noted in the Tables and Figures.

Results

Chromatographic separation of amino acid standard solution

A chromatogram of synthetic mixture of amino acid standards was received by RP-HPLC. Each peak represents one of specific amino acid, the peak was compact and symmetrical. 14 amino acids were completely separated (T<50 min) under the experimental conditions used, the peak sequence of 14 amino acids was: aspartic acid, glutamic acid, asparagine, serine, glutamine, glycine, arginine, alanine, tyrosine, valine, tryptophan, phenylalanine, isoleucine, leucine. We prepared the amino acid mixed standard solution at concentration of 31.25 - 500 μmol/L. After derivatization, we measured the samples. Taking the amino solution concentration (x) as the abscissa and corresponding peak area (y) as the coordinate. As shown in Table 4, in 31.25 - 500 μmol/L concentration range, amino acid standard concentration was linear related to the peak area and the correlation coefficients is 0.9960 - 0.9999. The Intra-day RSD and Inter-day RSD is between 2.15% - 4.05% and 3.13% - 4.83%, respectively, which are within 6%. These parameters results indicated that this sensitive procedure could be used for the quantitative analysis of amino acid in tissues.

Amino acid content in jugular venous plasma

As shown in Figure 1, all of the 14 kinds of amino acid concentrations were higher in buffering agent groups than that in the high grain group. The amino acid content of Gln showed very significant difference ($P < 0.01$) between these two groups. And the amino acid concentrations of Asn, Ser, Gly and Arg were significantly higher ($P < 0.05$) than that in high grain group.

Different types of amino acids in jugular venous plasma

As shown in Figure 2, the amino acid concentrations of TFAA, GAA and NEAA were significantly higher (P <0.05) than that in high grain group (2A). And the total AA content performed by Total Amino Acid assay kit in plasma of lactating goats showed

very significant difference (P <0.01) between these two groups (2B).

mRNA transcription level of amino acid transport

The excitatory amino acid transporter 1 (EAAT1, encoded by gene SLC1A3), alanine-serine-cysteine transporter 2 (ASCT2, encoded by gene SLC1A5), L-type amino acid transporter 1 (LAT1, encoded by gene SLC7A5), sodium-independent neutral and basic amino acid transporter (y^+LAT2, encoded by gene SLC7A6) and sodium-coupled neutral amino acid transporter 2 (SNAT2, encoded by gene SLC38A2) were reported as transporters of amino acids to maintain cell growth and protein synthesis in ruminant (19−21). However, the function of these amino acid transporters in regulating milk protein synthesis in the mammary gland of the lactating goats remains largely unknown. Therefore, mRNA expression levels of the corresponding genes encoding amino acid transporters in the mammary gland were detected by RT-PCR to reflect the role of these amino acid transporters in the mammary gland. SLC1A3, SLC1A5, SLC7A5, SLC7A6 and SLC38A2 mRNA expression levels in mammary tissues were up-regulated in BG goats compared to HG goats, the mRNA expressions of SLC38A2 was significantly increased (P < 0.05), and the mRNA expressions of SLC7A6 was very significantly increased (P < 0.01) in BG goats compared to HG goats (Figure 3).

Global identification of differentially expressed proteins in the mammary gland

Comparative proteomic analysis was performed between HG and BG lactating Saanen goats' mammary tissues, in order to understand the influence of adding buffering agent in high-grain diet on the mammary metabolism. As shown in Figure 4, an average of 1200 spots were detected on gels for both types of proteomes. We successfully found a total of 55 differential protein spots and 32 differential protein spots ($p < 0.05$; in terms of expression, all 32 with a fold change $\geqslant$1.5-fold) were successfully identificated in BG vs. HG using 2-DE technique and MALDI-TOF/TOF proteomics analyzer. Of these, 15 proteins showed increased expression and 17 proteins showed decreased expression in BG vs. HG respectively.

We can observe the main differential protein spots related to amino acid metabolism, glucose metabolism, lipid metabolism, oxidative stress, mitochondrial function, cytoskeletal structure and immune protein depicted in Table 5.

Beta casein (CSN2) is the main composition of milk protein. Lactoferrin (LF) is a nonheme iron binding glycoprotein in milk and a member of transferring family, during lactation expressed and secreted by the mammary epithelial cells at mucosal surface.

The protein expression of CSN2 and LF were up-regulated by 2-DE technique and MALDI-TOF/TOF proteomics analyser in BG as compared to the HG.

Validation of differentially expressed milk proteins

To further validation the CSN2 and LF proteins (main composition of milk protein), we performed western blot analysis for verification (Figure 5). The CSN2 protein expression level was extremely significant higher ($P < 0.01$) in BG than that in HG. And the LF protein expression level was also higher in BG than that in HG ($P < 0.05$).

The signaling pathway for milk protein synthesis

Western blot was applied to study the mechanism of the milk protein synthesis, the mTOR phosphorylation ratio and P70S6K phosphorylation ratio were significant up-regulated ($P < 0.05$, Figure 6B and 6C), the eIF4E phosphorylation ratio and the expression level of eEF2 were up-regulated, and showed significant difference ($P < 0.01$, Figure 6D and 6F), but the eEF2K phosphorylation ratio was down-regulated ($P < 0.01$, Figure 6E). It suggested that adding buffering agent could promote the protein expression levels of mTOR phosphorylation ratio, P70S6K phosphorylation ratio, eIF4E phosphorylation ratio and eEF2, and suppress the protein expression of eEF2K phosphorylation ratio.

Discussion

Milk production of dairy is determined mainly by the fat synthesis and milk protein and proliferation abilities of mammary epithelial cells (BMECs) (22). And milk proteins in the lactating ruminant mammary gland are primarily synthesized from circulating plasma amino acids, amino acids present in both free and peptide-bound forms, used by tissues both as building blocks for protein synthesis and as signaling molecules to regulate the protein synthetic machinery (23,24). The mammary gland requires large amounts of amino acids for the synthesis of milk protein. Uptake of amino acids from the arterial blood of the lactating dam is the ultimate source of proteins (primarily β-casein and α-lactalbumin) in milk (25). During lactation the ruminant mammary gland must import from the plasma and endogenously synthesize sufficient quantities of amino acids to elevate milk protein synthesis (26). Identifying and understanding changes of amino acids in plasma and and amino acids transport into the lactating mammary gland may provide fundamental knowledge towards the development of nutritional regimes aimed at elevating milk production. Here we found that compared with the HG, 14 kinds of free amino acid concentrations determined were higher in BG, and the amino acid concentrations of TFAA, GAA and NEAA were significantly higher

(P <0.05) than that in high grain group by HPLC. And the total AA concentration performed by Total Amino Acid assay kit in plasma of lactating goats also showed very significant difference (P <0.01) between these two groups. Therefore, adding buffering agent could promote the increase of amino acid concentration in blood compared with long-term feeding of high grain diet.

Presently, the amino acid transporter system in the mammary gland is not well-understood, but information suggests that the mammary gland has a transport system similar to other organs (intestine, kidney, placenta) (27). The future studies need to be conducted to identify changes in mammary genes involved in amino acid transport and uptake in the mammary gland. Main transporters involved in amino acid transport and uptake in lactating goat's mammary gland were investigated. SLC1A3, SLC1A5, SLC7A5, SLC7A6 and SLC38A2 mRNA expression levels in the mammary gland were detected by RT-PCR to reflect the role of these amino acid transporters in the mammary gland. We found that the mRNA expression levels of SLC1A3, SLC1A5, SLC7A5, SLC7A6 and SLC38A2 in mammary tissues were up-regulated in BG goats compared to HG goats, the mRNA expressions of SLC38A2 was significantly increased ($P < 0.05$), and the mRNA expressions of SLC7A6 was very significantly increased ($P < 0.01$) in BG goats compared to HG goats. EAAT1, encoded by SLC1A3 is one isoform of system X_{AG}^- and is a Na^+-dependent transporter with high affinity for Asp and Glu. ASCT2, encoded by SLC1A5 is Na^+-dependent, and has affinity for small neutral AA, such as Ala, Ser and Cys (28). y^+LAT2, encoded by SLC7A6 is a catalytic light chain (y^+LAT) and togethers with a heavy subunit (4F2hc) linked by a disulfide bond to form a heteromeric Na^+-dependent transporters y^+LAT2/4F2hc (Torrents et al. 1998) (29). y^+LAT2/4F2hc is located in the basolateral cell membrane, functions as obligatory asymmetric AA exchangers and has affinity for Lys, Arg, Gln, His, Met, Leu, Ala and Cys (30). LAT1, encoded by SLC7A5 found in many different types of mammalian cells, is indispensable as a transporter of essential AA to maintain cell growth and protein synthesis (31). SNAT2, encoded by SLC38A2 is expressed in the mammary gland and plays an important role in the uptake of alanine and glutamine which are the most abundant amino acids transported into this tissue during lactation, and has affinity for Gly, Pro, Ala, Ser, Cys, Gln, Met, His and Asn. The expression of SNAT2 can be upregulated by amino acids and hormones (prolactin and 17β-estradiol) (32). Our experimental data, combined with these reports, suggest that adding buffering agent could not only promote the increase of amino acid concentration in blood but also

337 through amino acid transporters transport more amino acids from the blood to the
338 mammary gland for the synthesis of milk protein compared with long-term feeding of
339 high grain diet.
340 Milk protein is mainly composed of casein and whey protein, the protein content in
341 milk from caprine species is approximately 2.1- 3.5%, caprine milk has a high casein
342 to whey ratio of 6.0, casein is synthesized by the epithelial cells of the mammary gland
343 (33). It is a group of phosphoproteins unique to milk and contains a large amount of
344 phosphorus and calcium. The casein fraction consists of α_{s1}-, α_{s2}-, β- and κ-casein. The
345 whey fraction consists of mainly β-lactoglobulin (β-lg), α-lactalbumin (α-la),
346 immunoglobulins, lactoferrin (LF) and lysozyme (34-36). In order to verify the amino
347 acids go into the mammary gland are more utilized by the mammary gland to synthesize
348 milk proteins, we used 2-DE technique and MALDI-TOF/TOF proteomics analyzer to
349 confirm it. We observed the protein expressions of CSN2 and LF were up-regulated by
350 2-DE technique and MALDI-TOF/TOF proteomics analyser in BG as compared to the
351 HG. Further verification by WB indicated that CSN2 protein expression level was
352 extremely significant higher ($P < 0.01$) in BG than that in HG. And the LF protein
353 expression level was also higher in BG than that in HG ($P < 0.05$).
354 It has been reported that β-casein has the highest content in casein of caprine milk,
355 which is different from the milk of bovine (37). Lactoferrin is a nonheme iron binding
356 glycoprotein in milk and a member of transferring family, during lactation expressed
357 and secreted by the mammary epithelial cells at mucosal surface. The results showed
358 that adding buffering agent in high grain diet promoted the synthesis of casein which is
359 the main components of milk protein and lactoferrin which comes from whey protein
360 in the mammary gland, so it was further verified that more milk protein, especially
361 casein and lactoferrin was synthesized by amino acid in blood entering into the
362 mammary gland.
363 It is widely accepted that mammalian target of rapamycin (mTOR) is variety of
364 components of protein synthesis and a key regulator of milk protein synthesis, most
365 reports were concerned with the role of amino acids and mTOR in protein synthesis, but
366 the mechanism of how amino acids enter the mammary gland to regulate the mTOR
367 signaling pathway and promote milk protein synthesis is still not very clear (38-40). To
368 further explore the mechanisms by which the buffering agent treatment regulated related
369 proteins expression in milk protein synthesis, we studied the activity of the mTOR
370 signaling pathway. In the present study, the results showed that the amino acids could

activate mTOR signaling which is consistent with previous reports. Here we identifies mTOR phosphorylation site as an indicator of activated mTOR pathway, which was increased by the increasing amino acids entering into mammary gland. mTORC1 activates P70S6K via phosphorylation, which in turn activates several proteins that contribute to increases in protein synthesis. In addition, activation of P70S6K inhibits eEF2K via phosphorylation, subsequently stimulating eEF2, which activates translocation elongation. When mTORC1 is phosphorylated it inhibits 4E-binding protein 1 (4EBP1) through phosphorylation, which inhibits eIF4E. eIF4E activates cap-dependent translocation increasing protein synthesis. Through these various proteins, mTORC1 stimulates cell growth by increasing cap-dependent translocation, translation elongation, mRNA biogenesis, and ribosome biogenesis, which leads to an increase in overall protein synthesis.

Conclusion

These results suggested that feeding of high-grain diet with buffering agent promoted the jugular vein blood of amino acids concentration, and the mRNA expressions of amino acid transport SLC1A3, SLC1A5, SLC7A5, SLC7A6 and SLC38A2 in mammary tissues were up-regulated, it showed that more amino acids flowed into the mammary. Two-dimensional electrophoresis and WB analysis further verification showed milk protein synthesis was increased. The results of the WB analysis suggested that the increase of milk protein synthesis was related to the activation of mTOR pathway signaling (Figure 7).

Abbreviations

HPLC: high performance liquid chromatography; SARA: subacute ruminal acidosis; CSN2: beta casein; LF: Lactoferrin

Acknowledgments

We are grateful to Hongyu Ma in Nanjing Agricultural University for advice on MS analysis. We also thank Xiangzhen Shen, Yingdong Ni and Su Zhuang (Nanjing Agricultural University) for their suggestions about the diet treatment.

Authors' contributions

MH conceived of the study, carried out the experiments and drafted the manuscript. XN and HW collected the sample and performed the research, analyzed data. MH and XN assisted with the sample analysis. YZ participated in the study's design and coordination. All authors read and approved the final manuscript.

Funding

This research was sponsored by grants from '13th Five-year Plan' National Key Research and Development Plan (Project No. 2017YFD0500505 and No. 2018 FYD 0601900).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. The protocols were reviewed and approved, and the project number 2011CB100802 was assigned. The slaughter and sampling procedures strictly followed the '*Guidelines on Ethical Treatment of Experimental Animals*' (2006) no. 398 established by the Ministry of Science and Technology, China and the '*Regulation regarding the Management and Treatment of Experimental Animals*' (2008) no. 45 set by the Jiangsu Provincial People's Government.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Dong, H.; Jia, Y.; Ni, Y.; Zhang, Y.; Zhuang, S.; Shen, X.; Zhao, R. Long-term effects of subacute ruminal acidosis (SARA) on milk quality and hepatic gene expression in lactating goats fed a high-concentrate diet. *Plos One*. **2013**, *8*, e82850.
2. Hook, S. E.; Steele, M. A.; Northwood, K. S.; Wright, A. G.; Mcbride, B. W. Impact of high-concentrate feeding and low ruminal pH on methanogens and protozoa in the rumen of dairy cows. *Microb Ecol*. **2011**, *62*, 94-105.
3. Alzahal, O.; Kebreab, E.; Mcbride, B. W. A mathematical approach to predicting biological values from ruminal pH measurements. *J Dairy Sci*.**2007**, *90*, 3777-3785.
4. Erdman, R. A.; Botts, R. L.; Hemken, R. W.; Bull, L. S. Effect of dietary sodium bicarbonate and magnesium oxide on production and physiology in early lactation. *J Dairy Sci*. **1980**, *63*, 923-930.
5. Kurokawa, Y.; Shibata, H.; Tateno, S.; Kanda, S.; Takaura, K.; Ishida, S.; Itabashi, H. Rumen fermentation, milk production and conjugated linoleic acid in the milk of cows fed high fiber diets added with dried distillers grains with solubles. *Anim Sci J*. **2013**, *84*, 106-112.
6. Bhuiyan, A.; Carotenuto, F.; Vitale, E.; Mauretti, A.; Neri, A.; Nanni, F.; Nardo, P. D.; Melinc, S. Abstracts presented at the 13th international congress on amino acids, peptides and proteins. *Amino Acids*. **2013**, *45*, 563-612.
7. Russell, T. D.; Palmer, C. A; Orlicky, D. J.; Fischer, A.; Rudolph, M. C.; Neville, M. C.;

Mcmanaman, J. L. Cytoplasmic lipid droplet accumulation in developing mammary epithelial cells: roles of adipophilin and lipid metabolism. *J Lipid Res*. **2007**, *48*, 1463-1475.

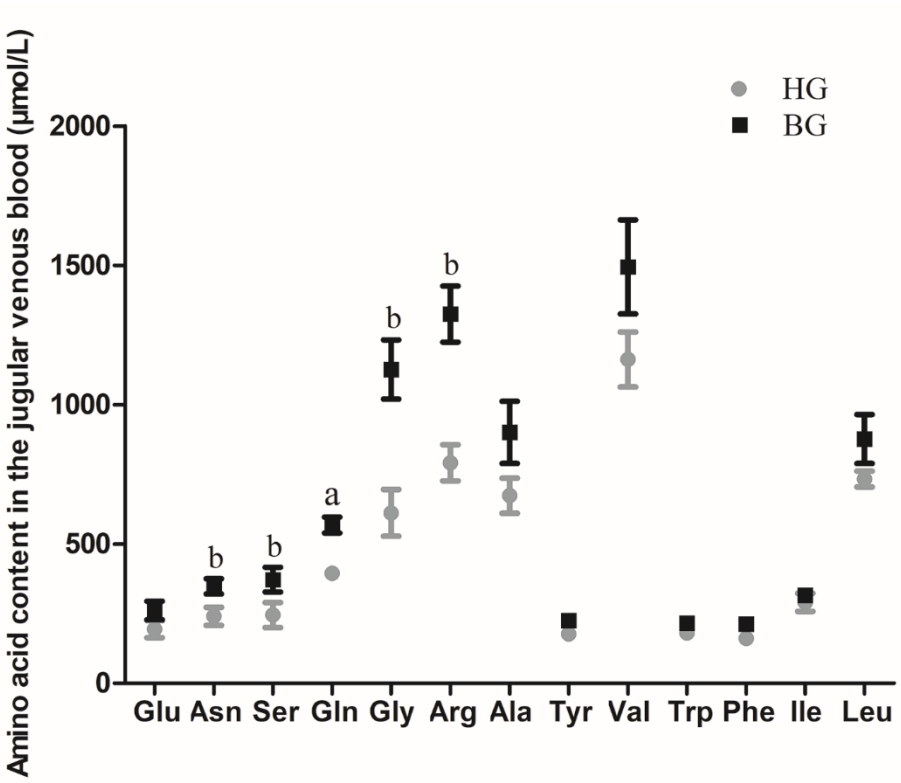
8. Yu, Q.; Zhu, L.; Lin, J.; Zhang, Q.; Tian, Q.; Hu, w.; Yang, Q. Functional analyse of GLUT1 and GLUT12 in glucose uptake in goat mammary gland epithelial cells. *Plos One*. **2013**, *8*, e65013.
9. Osorio, J. S.; Lohakare, J.; Bionaz, M. Biosynthesis of milk fat, protein, and lactose: roles of transcriptional and posttranscriptional regulation. *Physiol Genomics*. **2016**, *48*, 231-256.
10. Shen, F.; Niu, X.; Yang, D.; Ying, Y.; Li, B.; Zhu, G.; Wu, J. Determination of amino acids in chinese rice wine by fourier transform near-infrared spectroscopy. *J Agric Food Chem*. **2010**, *58*, 9809-9816.
11. Han, N.; Li, L.; Peng, M.; Ma, H. Hydroxycitric acid nourishes protein synthesis via altering metabolic directions of amino acids in male rats. *Phytother Res*. **2016**, *30*, 1316-1329.
12. Duanmu, Y.; Cong, R.; Tao, S.; Tian, J.; Dong, H.; Zhang, Y.; Ni, Y.; Zhao, R. Comparative proteomic analysis of the effects of high-concentrate diet on the hepatic metabolism and inflammatory response in lactating dairy goats. *J Anim Sci Biotechno*. **2016**, *7*, 1-11.
13. Shen, F.; Niu, X.; Yang, D.; Ying, Y.; Li, B.; Zhu, G.; Wu, J. et al. Determination of amino acids in chinese rice wine by fourier transform near-infrared spectroscopy. *J Agric Food Chem*. **2010**, *58*, 9809-9816.
14. Chen, J.; Tang, X.; Zhang, Y.; Ma, H.; Zou, S. Effects of maternal treatment of dehydroepiandrosterone (DHEA) on serum lipid profile and hepatic lipid metabolism-related gene expression in embryonic chickens. *Comp Biochem Phys B*. **2010**, *155*, 380-386.
15. Zeng, T.; Jiang, X.; Li, J.; Wang, D.; Li, G.; Lu, L.; Wang, G. Comparative proteomic analysis of the hepatic response to heat stress in muscovy and pekin ducks: insight into thermal tolerance related to energy metabolism. *Plos One*. **2013**, *8*, e76917.
16. Huang, J.; Gao, X.; Li, Q.; Lu, L.; Liu, R.; Luo, C.; Wang, J.; Bin, Q.; Jin, X. Proteomic analysis of the nuclear phosphorylated proteins in dairy cow mammary epithelial cells treated with estrogen. *In Vitro Cell Dev-An*. **2012**, *48*, 449-457.
17. Ura, B.; Scrimin, F.; Arrigoni, G.; Franchin, C.; Monasta, L.; Ricci, G. A proteomic approach for the identification of up-regulated proteins involved in the metabolic process of the leiomyoma. *Int J Mol Sci*. **2016**, *17*, 540.
18. Dai, B.; Zhang, J.; Liu, M.; Lu, J.; Zhang, Y.; Xu, Y.; Miao, J.; Yin, Y. The role of Ca(2+) mediated signaling pathways on the effect of taurine against Streptococcus uberis infection. *Vet Microbiol*. **2016**, *192*, 26-33.
19. Kanai, Y.; Segawa, H.; Miyamoto, K.; Uchino, H.; Takeda, E.; Endou, H. Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). *J Biol Chem*. **1998**, *273*, 23629-23632.
20. Baumrucker, C. R. Cationic amino acid transport by bovine mammary tissue. *J Dairy Sci*. **1984**, *67*, 2500-2506.

21. Broer, A.; Rahimi, F.; Broer, S. Deletion of amino acid transporter ASCT2 (SLC1A5) reveals an essential role for transporters SNAT1 (SLC38A1) and SNAT2 (SLC38A2) to sustain glutaminolysis in cancer cells. *J Biol Chem*. **2016**, *291*, 13194-13205.
22. Herve, L.; Quesnel, H.; Lollivier, V.; Boutinaud, M. Regulation of cell number in the mammary gland by controlling the exfoliation process in milk in ruminants. *J Dairy Sci*. **2016**, *99*, 854-863.
23. Sancak, Y.; Barpeled, L.; Zoncu, R.; Markhard, A. L.; Nada, S.; Sabatini, D. M. Ragulator-rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell*. **2010**, *141*, 290-303.
24. Wang, Y.; Liu, J.; Wu, H.; Fang, X.; Chen, H.; Zhang, C. Amino acids regulate mTOR pathway and milk protein synthesis in a mouse mammary epithelial cell line is partly mediated by T1R1/T1R3. *Eur J Nutr*. **2017**, *56*, 1-8.
25. Rezaei, R.; Wu, Z.; Hou, Y.; Bazer, F. W.; Wu, G. Amino acids and mammary gland development: nutritional implications for milk production and neonatal growth. *J Anim Sci Biotechnol*. **2016**, *7*, 20-42.
26. Lapierre, H.; Lobley, G. E.; Doepel, L.; Raggio, G.; Rulquin, H.; Lemosquet, S. Triennial lactation symposium: mammary metabolism of amino acids in dairy cows. *J Anim Sci*. **2012**, *90*, 1708-1721.
27. Lei, J.; Feng, D.; Zhang, Y.; Zhao, F.; Wu, Z.; San, G. A.; Fujishima, Y.; Uneyama, H.; Wu, G. Nutritional and regulatory role of branched-chain amino acids in lactation. *Front Biosci*. 2012, 17, 2725-2739.
28. Manjarin, R.; Bequette, B. J.; Wu, G.; Trottier, N. L. November 2014, Linking our understanding of mammary gland metabolism to amino acid nutrition. *Amino Acids*. **2014**, *46*, 2447-2462.
29. Torrents, D.; Estevez, R.; Pineda, M.; Fernandez, E.; Lloberas, J.; Shi, Y.; Zorzano, A.; Palacıin, M. Identification and characterization of a membrane protein (y+L amino acid transporter-1) that associates with 4F2hc to encode the amino acid transport activity y+L a candidate gene for lysinuric protein intolerance. *J Biol Chem*. **1998**, *273*, 32437-32445.
30. Yoshikatsu Kanai, Y.; Fukasawa, Y.; Cha, S. H.; Segawa, H.; Chairoungdua, A.; Kim, D. K.; Matsuo, H.; Kim, J. Y.; Miyamoto, K.; Takeda, E.; Endou, H. Transport properties of a system y+L neutral and basic amino acid transporter: Insights into the mechanisms of substrate recognition. *J Biol Chem*. **2000**, *275*, 20787-20793.
31. Lin, Y.; Duan, X.; Lv, H.; Yang, Y.; Liu, Y.; Gao, X.; Hou, X. The effects of L-type amino acid transporter 1 on milk protein synthesis in mammary glands of dairy cows. *J Dairy Sci*. **2018**, *101*, 1687-1696.
32. Velazquez, L. A.; Ortiz, V.; Strom, A.; Torres, N.; Engler, D. A.; Matsunami, R.; Ordaz, D.; Garcia, R.; Lopez, A. M.; Larrea, F.; Gustafsson, J. A.; Tovar, A. R. Transcriptional regulation of the sodium-coupled neutral amino acid transporter (SNAT2) by 17β-estradiol. *Proc Natl*

Acad Sci USA. **2014**, *111*, 11443-11448.

33. Miranda, G.; Mahe, M. F.; Leroux, C.; Martin, P. Proteomic tools to characterize the protein fraction of equidae milk, *Proteomics*. **2004**, *4*, 2496-2509.
34. Inglingstad, R. A.; Devold, T. G.; Eriksen, E. K.; Holm, H.; Jacobsen, M.; Liland, K. H.; Rukke, E. O.; Vegarud, G. E. Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. *Dairy Sci Technol*. **2010**, *90*, 549-563.
35. Park, Y. W.; Juarez, M.; Ramos, M.; Haenlein, G. F. W. Physico-chemical characteristics of goat and sheep milk. *Small Ruminant Res*. **2007**, *68*, 88-113.
36. Jenness, R. Composition and characteristics of goat milk: review 1968–1979. *J Dairy Sci*.1980, *63*, 1605-1630.
37. Selvaggi, M.; Laudadio, V.; Dario, C.; Tufarelli, V. Major proteins in goat milk: an updated overview on genetic variability. *Mol Biol Rep*. **2014**, *41*, 1035-1048.
38. Arriola, S. I.; Bell, A. L.; Estes, K.; Ropelewski, J.; Veth, M. J.; Hanigan, M. D. Effects of reduced dietary protein and supplemental rumen-protected essential amino acids on the nitrogen efficiency of dairy cows. *J Dairy Sci*. **2014**, *97*, 5688-5699.
39. Dai, W. T.; White, R. R.; Liu, J. X.; Liu, H. Y. Seryl-tRNA synthetase-mediated essential amino acids regulate β-casein synthesis via cell proliferation and mammalian target of rapamycin (mTOR) signaling pathway in bovine mammary epithelial cells. *J Dairy Sci*. **2018**, *101*, 10456-10468.
40. Li, S.; Loor, J. J.; Liu, H.; Liu, L.; Hosseini, A.; Zhao, W.; Liu, J. Optimal ratios of essential amino acids stimulate β-casein synthesis via activation of the mammalian target of rapamycin signaling pathway in MAC-T cells and bovine mammary tissue explants. *J Dairy Sci*. **2017**, *100*, 6676-6688.
41.

Figure 1



543

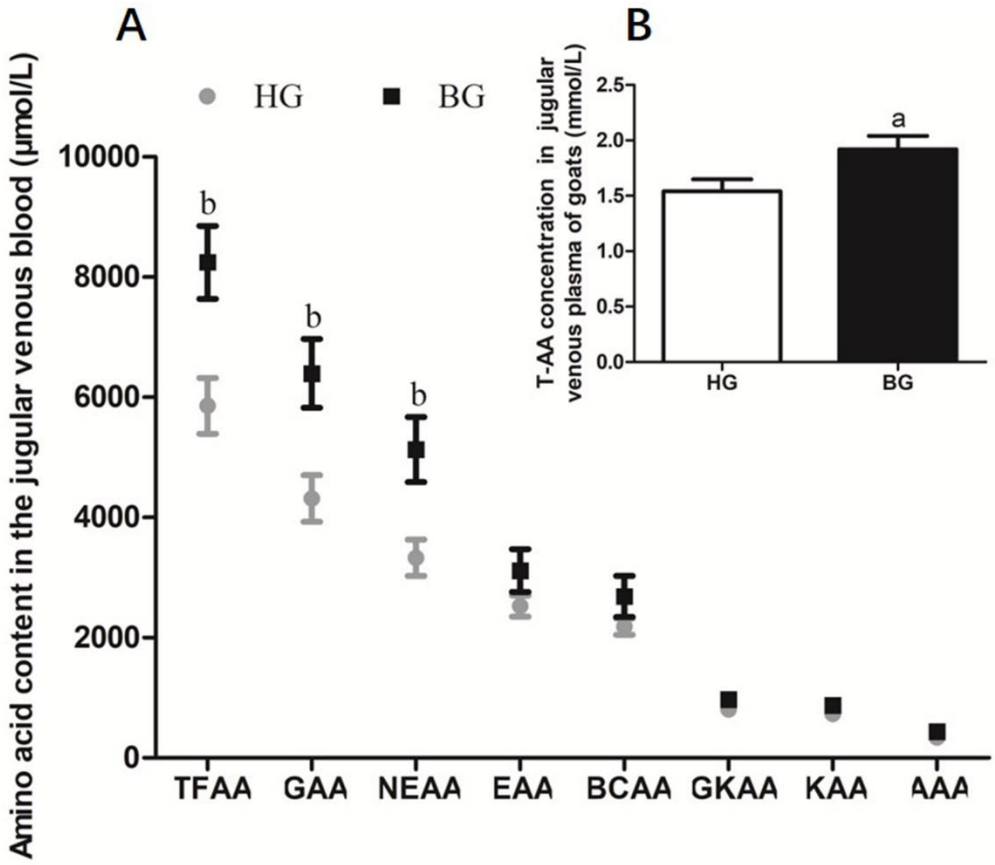
544 **Figure 1 Impact of high-grain diet with buffering agent on amino acid content in plasma of**

545 **lactating goats.**

546 Values are mean ± SEM, n = 6/group. $^{b}p<0.05$ and $^{a}p<0.01$, compared with high grain group.

547

548 **Figure 2**



549

550 **Figure 2 Different types of amino acids content in plasma of lactating goats.**

551 (A) Impact of high-grain diet with buffering agent on different types of amino acids content in
552 plasma of lactating goats by HPLC. (B) Impact of high-grain diet with buffering agent on total
553 amino acids content in plasma of lactating goats by Total Amino Acid assay kit. Values are mean ±
554 SEM, n = 6/group. $^{b}p<0.05$ and $^{a}p<0.01$, compared with high grain group. TFAA, total free amino
555 acid; GAA, glycogenic amino acid; NEAA, non-essential amino acid; EAA, essential amino acid;
556 BCAA, branched-chain amino acid; GKAA, glucogenic and ketogenic amino acid; KAA, ketogenic
557 amino acid; AAA, aromatic amino acid.

558

559 **Figure 3**

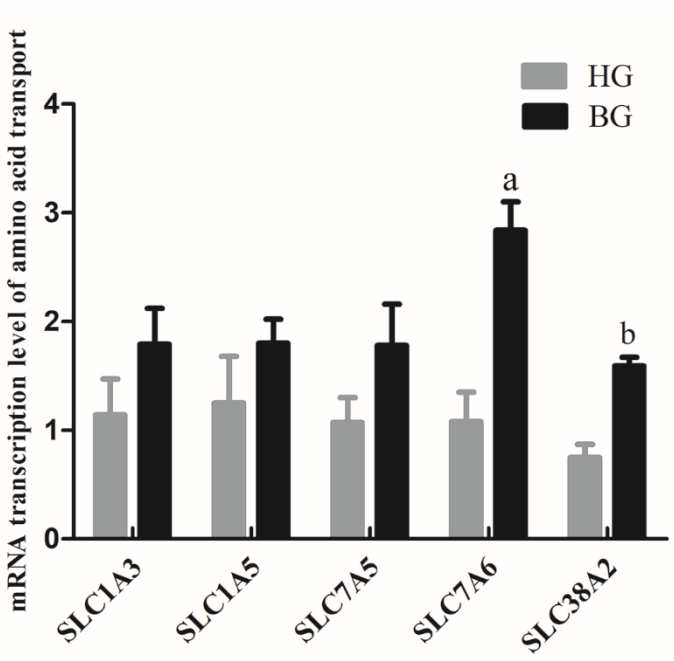


Figure 3 mRNA expressions of amino acid transport SLC1A3, SLC1A5, SLC7A5, SLC7A6 and SLC38A2.

Each sample was first normalized against its GAPDH transcript level, and then normalized to the HG. In order to calculate differences in the expression level of each target gene, the $2^{-\Delta\Delta Ct}$ method for relative quantification was used, according to the manufacturer's manual. Values are mean ± SEM. n = 6/group. $^{b}p<0.05$ and $^{a}p<0.01$, compared with high grain group.

Figure 4

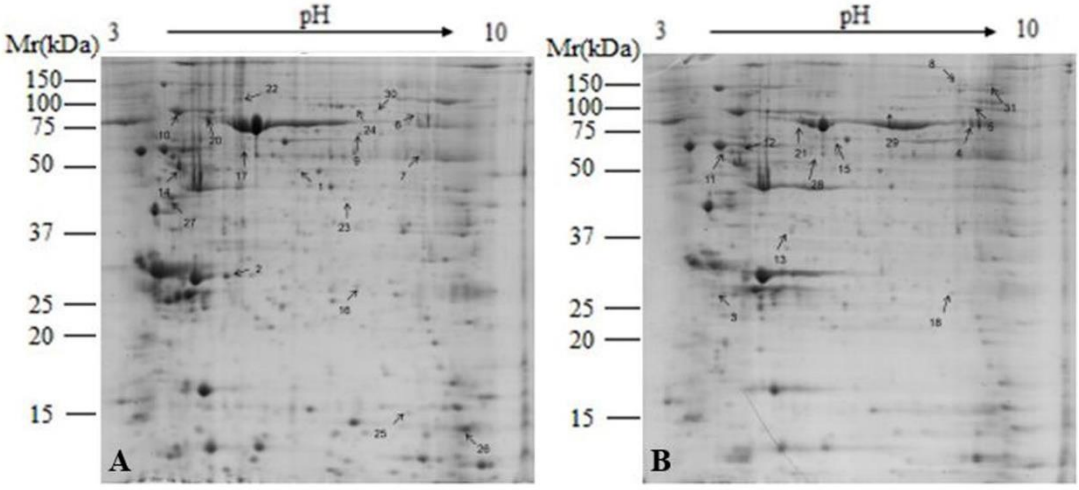


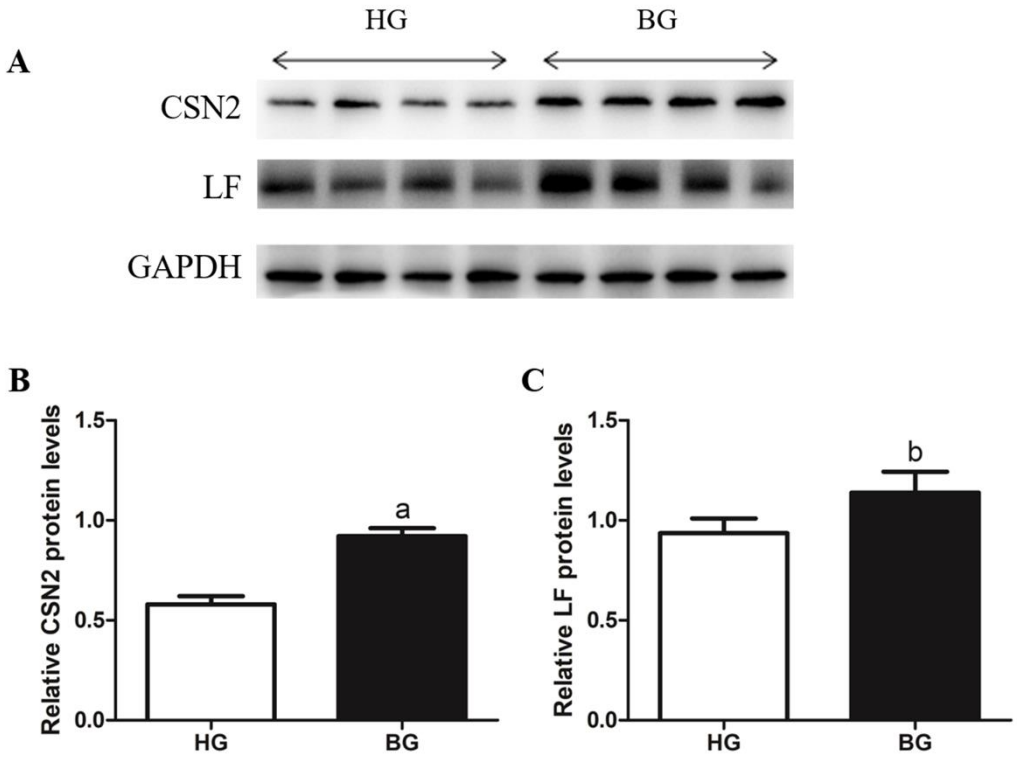
Figure 4 Representative 2-DE images of proteins extracted from lactating goat mammary gland.

(A) High-grain group; (B) Buffering agent group. Equal amounts of protein (1000 mg) were loaded onto the 17 cm IPG gel strip (nonlinear, pH 3.0-10.0), and separated on 17-cm IPG strips (pH 3.0-10.0), followed by electrophoresis on 12.5% SDS-PAGE gels for second dimension electrophoresis.

575 Black arrows indicate differential protein spots (≥1.5-fold).

576

577 **Figure 5**



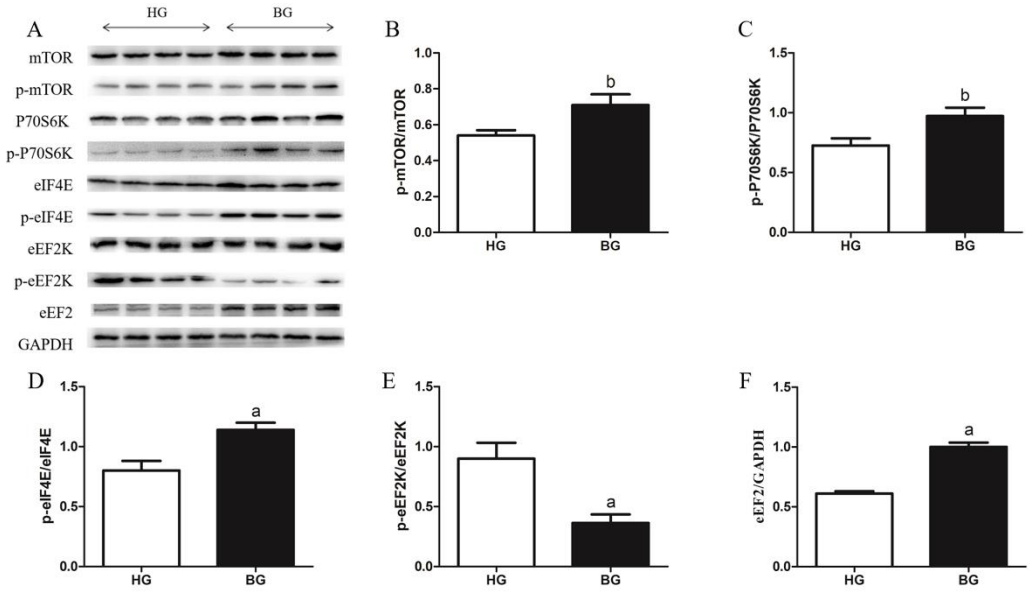
578

579 **Figure 5 Western blot analysis of the CSN2 and LF.**

580 (A) Indicated protein levels were measured by western blotting analysis. (B and C) Relative protein
581 levels of CSN2 (B) and LF (C) from the western blots were quantified by gray scale scan. Values
582 are mean ± SEM. n = 4/group. $^{b}p<0.05$ and $^{a}p<0.01$, compared with high grain group.

583

584 **Figure 6**



585

Figure 6 Milk protein synthesis mTOR related signaling pathway protein expressions.

(A) mTOR related signaling pathway protein expression levels were measured by western blotting analysis. (B) The ratios of phosphorylated to total mTOR were quantified by gray scale scan. (C) The ratios of phosphorylated P70S6K to total P70S6K were quantified by gray scale scan. (D) The ratios of phosphorylated eIF4E to total eIF4E were quantified by gray scale scan. (E) The ratios of phosphorylated eEF2K to total eEF2K were quantified by gray scale scan. (F) Relative protein levels of eEF2 from the western blots were quantified by gray scale scan. Values are mean ± SEM. n = 4/group. $^{b}p<0.05$ and $^{a}p<0.01$, compared with high grain group.

Figure 7

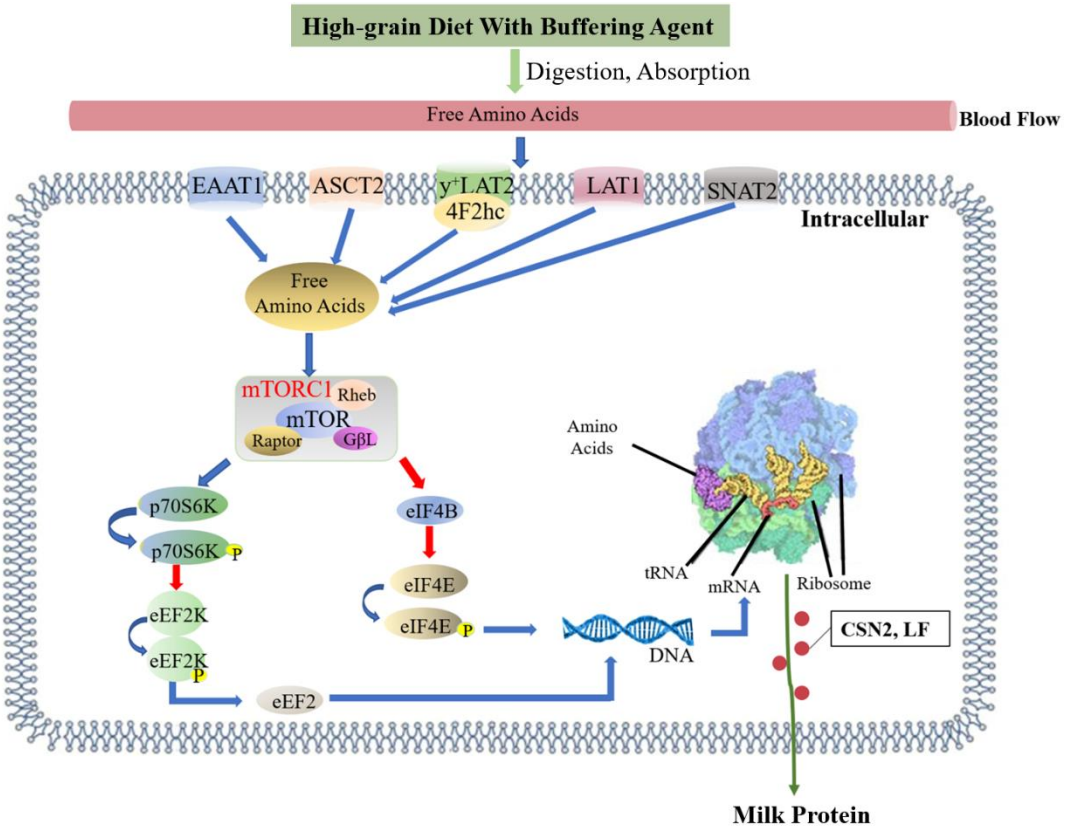


Figure 7 Effects of high-grain diet with buffering agent on the milk protein synthesis.

Feeding of high-grain diet with buffering agent promoted the jugular vein blood of amino acids concentration, and the mRNA expressions of amino acid transport SLC1A3, SLC1A5, SLC7A5, SLC7A6 and SLC38A2 in mammary tissues were up-regulated, it showed that more amino acids flowed into the mammary. Amino acids in mammary gland activated mTOR pathway. mTORC1 activates P70S6K via phosphorylation, which in turn activated several proteins that contributed to promote protein synthesis. In addition, activation of P70S6K inhibited eEF2K via phosphorylation, subsequently stimulated eEF2, which activated translocation elongation. When mTORC1 was phosphorylated, it inhibited 4EBP1 through phosphorylation, which inhibits eIF4E. eIF4E activates cap-dependent translocation increasing protein synthesis. The blue arrow represents promotion, and the red arrow represents inhibition.

608

609 **Table**

610 Table 1. Ingredients and nutritional composition of the diets.

Concentrate: Forage ratio 60:40			
Ingredient (%)		Nutrient levels[b]	
Leymus chinensis	27.00	Net energy/(MJ.kg^{-1})	6.71
Alfalfa silage	13.00	Crude protein/%	16.92
Corn	23.24	Neutral detergent fiber/%	31.45
Wheat bran	20.77	Acid detergent fiber/%	17.56
Soybean meal	13.67	Calcium/%	0.89
Limestone	1.42	Phosphorus/%	0.46
NaCl	0.30		
Premix[a]	0.60		
Total	100.00		

611 a. Provided per kg of diet: VA 6000IU/kg, VD 2500IU/kg, VE 80mg/kg, Cu 6.25 mg/kg, Fe 62.5
612 mg/kg, Zn 62.5 mg/kg, Mn 50mg/kg, I 0.125 mg/kg, Co 0.125 mg/kg.
613 b. Nutrient levels were according to National Research Council (NRC,2001).

614

615 Table 2. Gradient elution program of RP-HPLC.

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)	Flow rate (mL/min)
0	85	6	9	1
10	80	8	12	1
25	70	15	15	1
40	45	25	30	1
50	45	25	30	1

616

617 Table 3. Primer sequences used for qRT-PCR analysis of target genes in lactating goats.

Target genes	Primer sequences (5'→3')	Products/bp
SLC1A3	CATCGTGCTGACATCTGTGG/ CCATTTCGACATCCCGGTTC	173
SLC1A5	CATCAACGACTCTGTTGTAGACC / CGCTGGATACAGGATTGCGG	182
SLC7A5	GAGCGACCCATCAAGGTTCA / ACCGTCGTGGAAAAGATGCT	206

SLC7A6	AGCAGTGTGGGAAGTAAGGC/ CTGACGCCATTCAGCAGAGA	207
SLC38A2	AGTTCAGTTGGTGGCGTCAT / CGGTCATCACCACTATGCCA	243
GAPDH	GGGTCATCATCTCTGCACCT/ GGTCATAAGTCCCTCCACGA	180

618

Table 4. Determination parameters of standard amino acids.

Amino acids	Retention time (t/min)	Regression equation (μmol/L)	Correlation coefficients Intra-day (r)	Intra-day RSD (%)	Inter-day RSD (%)
Asp	6.025	y = 1.0518x - 7.5833	r=0.9998	2.52	3.14
Glu	8.624	y = 1.1656x - 13.771	r=0.9996	3.26	4.01
Asn	16.108	y = 0.5021x + 4.6208	r=0.9992	2.15	3.17
Ser	18.58	y = 0.8926x - 6.2708	r=0.9992	3.23	3.92
Gln	20.227	y = 0.8177x + 1.5174	r=0.9992	3.06	3.98
Gly	23.661	y=1.1565x- 11.778	r=0.9987	2.88	3.16
Arg	25.031	y=1.271x- 21.429	r=0.9974	2.69	3.64
Ala	30.285	y = 0.9363x - 8.8435	r=0.9988	2.67	3.13
Tyr	32.45	y=1.546x- 10.704	r=0.9985	3.59	3.78
Val	39.702	y = 1.7831x - 2.5696	r=0.9990	3.23	4.23
Trp	40.734	y = 1.3565x - 48.778	r=0.9979	3.18	3.67
Phe	42.086	y = 1.6486x - 45.243	r=0.9975	3.89	4.35
Ile	43.492	y = 1.7844x - 21.226	r=0.9977	4.01	4.54
Leu	44.521	y = 1.0609x + 7.7087	r=0.9986	4.05	4.83

Note: Asp, aspartic acid; Glu, glutamic acid; Asn, asparagines; Ser, serine; Gln, glutamine; Gly, glycine; Arg, arginine; Ala, alanine; Tyr, tyrosine; Val, valine; Trp, tryptophan; Phe, phenylalanine; Ile, isoleucine; Leu, leucine. RSD, relative standard deviation. The peak times of Met and Val, Lys and Tyr are almost the same, so Val and Tyr are selected in this experiment, Met and Lys are removed. The peak times of Thr and Arg are very close, and compared with Arg, the peak of Thr is very low and can not be completely separated, so Arg is selected. Cys peak is very low and the quantitative error is large, so it is also removed. Under this condition, His has two peaks, the exact peak time can not be determined, so the His is removed. Pro does not contain primary ammonia, and the derivatizing agent phthalaldehyde cannot be derivatized, so it cannot be determined.

619

Table 5. Differential expression protein spots by MAIDI-TOF-TOF.

Spot no.	Identified protein name	Gene symbol	Accession no.	Mr(kDa)	pI	Protein expression	1.5Fold change
Amino acid metabolism							
1	Ornithine aminotransferase	OAT	gi|803207295	37.8	8.37	Down	0.65
2	Beta casein	CSN2	gi|1211	24.9	5.26	Up	2.73
3	Peptidylproly l isomerase A	PPIA	gi|410066839	18.0	8.34	Down	0.66

4	Lactoferrin	LF	gi|56544486	79.2	8.4	Up	2.27
Glucose Metabolism							
5	Pyruvate kinase PKM isoform X7	PKM	gi|426232638	58.5	7.60	Down	0.23
6	UTP--glucose-1-phosphate uridylyltransferase isoform X1	UGP2	gi|426223460	57.0	7.68	Up	2.13
7	Transketolase	TKT	gi|426249391	68.5	7.2	Up	2.52
8	Aconitate hydratase, mitochondrial isoform X1	ACO2	gi|803058827	95.9	6.78	Down	0.5
9	Aldehyde dehydrogenase, mitochondrial	ALDH2	gi|426247368	57.1	7.55	Down	0.52
10	Glucose-regulated protein	HSPA5	gi|426223038	72.5	5.07	Up	1.66
Cytoskeletal structure							
11	Tubulin	Tubulin	gi|257097261	50.2	4.78	Up	1.66
12	Vimentin	VIM	gi|145226795	53.7	5.02	Up	1.56
13	F-actin-capping protein subunit beta isoform X1	CAPZB	gi|426222052	34.0	5.82	Down	0.49
14	Protein disulfide-isomerase A6	PDIA6	gi|803229999	48.5	4.95	Up	2.1
Lipid metabolism							
15	Glycerol kinase isoform X5	GK	gi|426256814	61.4	5.60	Down	0.41
16	Acid synthase isoform X2	FASN	gi|803208475	27.6	5.93	Up	6
Oxidative stress							
17	Cytochrome b-c1 complex subunit 1	UQCRC1	gi|803186314	51.9	6.03	Down	0.42
18	Glutathione S-transferase P	GSTP1	gi|803225108	23.8	6.89	Up	1.96
Mitochondrial function							
19	ATP synthase subunit beta, mitochondrial	ATP5B	gi|426224929	56.2	5.15	Up	1.66
20	NADH-ubiquinone oxidoreductase 75 kDa subunit	NDUFS1	gi|426221412	80.4	5.90	Down	0.46
Immune protein							
21	Pre-pro serum albumin	ALB	gi|1387	71.1	5.80	Down	0.18
22	Polymeric immunoglobulin	PIGR	gi|426239425	83.7	5.79	Down	0.17

	receptor						
23	Annexin A1	ANXA1	gi|426220300	39.0	6.17	Down	0.47
24	Serotransferrin isoform X2	TF	gi|803335974	79.4	6.31	Up	2.38
25	Beta globin chain	HBB	gi|86129745	16.0	6.75	Up	1.89
26	Alpha globin chain	HBAI	gi|1787	15.3	8.72	Up	1.60
Other							
27	Protein FAM186B isoform X1	FAM186B	gi|803043984	111.3	8.87	Down	0.47
28	T-complex protein 1 subunit epsilon	CCT5	gi|426246714	60.03	5.6	Down	0.57
29	Radixin isoform X1	RDX	gi|803154243	68.4	6.03	Down	0.53
30	Lamin isoform X2	LMNA	gi|803255736	74.4	6.76	Down	0.48
31	Elongation factor 2 isoform X2	EEF2	gi|426229147	96.2	6.41	Down	0.36
32	cytochrome b-c1 complex subunit 2	UQCRC2	gi|426254425	48.8	8.89	Up	2.47

620