Long-Term Effects of Subacute Ruminal Acidosis (SARA) on Milk Quality and Hepatic Gene Expression in Lactating Goats Fed a High-Concentrate Diet

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Abstract

Purpose: The mechanism underlying the decline in milk quality during periods of feeding high-concentrate diets to dairy ruminants is not well documented. The aim of this study was to investigate the metabolic changes in the liver that contribute to the input of substrate precursors to the mammary gland after feeding a high-concentrate diet to lactating goats for a long period.

Experimental Design: Eight mid-lactating goats with rumen cannulas were randomly assigned to two groups. For 9 weeks, the treatment group was fed a high-concentrate diet (60% concentrate of dry matter, HC) and the control group was fed a low-concentrate diet (40% concentrate of dry matter, LC). Ruminal fluid, plasma, and liver tissues were sampled, microarray techniques and real-time polymerase chain reaction were used to evaluate metabolic parameters and gene expression in liver.

Results: Feeding a 60%-concentrate diet for 9 weeks resulted in a significant decrease in rumen pH. Changes in fat and protein content also occurred, which negatively affected milk quality. Plasma levels of leptin \( p = 0.058 \), non-esterified fatty acid \( p = 0.071 \), and glucose \( p = 0.014 \) increased markedly in HC group. Plasma cortisol concentration was significantly elevated in the treatment group \( p < 0.05 \). Expression of the glucocorticoid receptor protein gene was significantly down-regulated \( p < 0.05 \) in the liver. The expression of genes for interleukin 1β, serum amyloid A, C-reactive protein, and haptoglobin mRNA was significantly increased \( p < 0.05 \) in the HC group. GeneRelNet analysis showed that gene expression involved in inflammatory responses and the metabolism of lipids, protein, and carbohydrate were significantly altered by feeding a high-concentrate diet for 9 weeks.

Conclusions: Activation of the acute phase response and the inflammatory response may contribute to nutrient partitioning and re-distribution of energy in the liver, and ultimately lead to a decline in milk quality.

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Introduction

Dairy ruminants are often fed a high-concentrate diet to meet the energy requirements of high milk production. However, metabolic disorders such as subacute ruminal acidosis (SARA) can result from feeding excessive amounts of high proportions of fermentable concentrate and forage with low physiologically effective fiber [1]. SARA is a common digestive disorder in most dairy herds. In intensive management systems, 19% of early lactation and 26% of mid-lactation cows can be affected by SARA [2]. The consequences of SARA include depression of food intake, diarrhea, laminitis, and inflammatory response, which ultimately result in the depression of milk quality and quantity [3] [4] [5] [6].
Fairfield et al. [7] and Khafipoor [8] reported that experimentally induced SARA decreased milk fat percentage but increased milk protein content. However, other reports have suggested that during the short term SARA did not affect milk fat or protein content [9] [10]. However, dairy production herds affected by SARA can experience negative outcomes as long as several weeks (>3 weeks) after an episode [11]. These diseases include laminitis, weight loss, and unexplained abscesses. The long-term metabolic effects of SARA have not been well studied.

Feeding high-concentrate diets to lactating cows results in the release of bacterial endotoxins, such as lipopolysaccharide (LPS), from the rumen or hind gut [12]. Free LPS can translocate into the bloodstream from the digestive tract under conditions of high permeability and after injury to the epithelial tissue [12]. Immune responses are then activated and pro-inflammatory cytokines are released from toll-like receptors 4 (TLR-4) that recognize the circulating LPS [13]. The process of LPS recognition via TLR-4 is facilitated by the accessory molecule LPS-binding protein (LBP) and cluster of differentiation antigen 14 (CD14) [14] [15]. LBP is an acute phase protein (APP). The acute phase response to infection, tissue injuries, or other disorders participates in the restoration of physiological homeostasis [16]. The liver is the major site for the synthesis of APPs, including LBP, C-reactive protein (CRP), serum amyloid A (SAA), and haptoglobin (HP) [17] [18] [19]. Monokines such as interleukin 6 (IL-6), IL-1, and tumor necrosis factor alpha (TNF-α) induce APP synthesis via their specific hepatic receptors [20]. These monokines also stimulate the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH signals an increased secretion of glucocorticoid hormone from the adrenal cortex [21]. During the restoration of physiological homeostasis, these mediators act on specific receptors on different target cells, which results in changes in metabolism and systemic responses.

As the vital organ that controls metabolism [22], the liver is stimulated by endogenous and exogenous factors. It is responsible for nutrient partitioning, including homeostatic and long-term homeorhetic adaptations [23]. Physical and psychological stress elevates plasma IL-6 and APP levels in humans and in experimental animals [24] [25]. Although the mechanism of APP induction in response to stress is yet to be elucidated, activation of the hypothalamic–pituitary–adrenal (HPA) axis by stressors may trigger systemic or local cytokine production. It’s reported that, during stress response physiological processes are aimed on redistribution of energy utilization in specific organs.

### Table 1. Comparison of plasma concentrations of glucose, non-esterified fatty acids (NEFA), triglyceride (TG) and leptin.

| Parameters     | LC (mmol/L) | HC (mmol/L) | P-value |
|----------------|-------------|-------------|---------|
| Glucose        | 3.00 ± 0.06 | 3.3 ± 0.05  | 0.014   |
| NEFA (mmol/L)  | 175.87 ± 55.12 | 969.52 ± 293.20 | 0.071   |
| TG (mmol/L)    | 0.19 ± 0.06 | 0.18 ± 0.03  | 0.659   |
| Leptin (ng/mL) | 1.75 ± 0.57 | 2.01 ± 0.78  | 0.058   |

LC, low concentrate; HC, high concentrate; NEFA, non-esterified fatty acid. TG, triglyceride. Values are mean ± standard error of the mean (SEM), n = 4/group. doi:10.1371/journal.pone.0082850.t001

**Figure 1. Rumen fluid pH values.** Data were analyzed for differences due to diet, time, and their interactions by Univariate using the General Linear Models of SPSS 11.0 for Windows (StatSoft Inc, Tulsa, OK, USA). Values are mean ± standard error of the mean (SEM). doi:10.1371/journal.pone.0082850.g001
Results

Ruminal fluid pH

After 1 week of feeding a high-concentrate diet, the mean ruminal pH value in the HC group was pH 5.8 from 1 to 5 hours after feeding, which was consistent with the experimental definition of SARA (Figure 1A). During week 4 to week 8, enhanced ruminal buffering capacity and diet adaptation resulted in an increase in pH of HC group (mean pH >6.0), but it was still lower than that of LC group (Figure 1B, C and D).

Plasma biochemical parameters and milk composition

In HC goats compared with LC counterparts, the levels of plasma non-esterified fatty acid (NEFA; \( p = 0.071 \)) had a tendency to increase and blood glucose \( (p = 0.014) \) was significantly increased after feeding high concentrate diet for 9 weeks (Table 1).

The percentage of milk fat and protein decreased significantly from 5–9 weeks in HC group compared with LC group \( (p<0.05) \). However, milk lactose concentration increased significantly in HC group from the 4th week of treatment compared to LC group \( (p<0.05; \) Table 2).

| Item                  | Diet  | Time       | Effect, P-value |
|-----------------------|-------|------------|-----------------|
|                      |       | 0–2 W     | 3–4 W           | 5–6 W           | 7–9 W           | Diet  | Time | Diet × Time |
| Fat                   | LC    | 4.15±0.43 | 4.01±0.38       | 3.76±0.88       | 3.90±0.53       |       |      |            |
|                       | HC    | 3.09±0.63*| 3.34±1.15       | 3.20±1.22*      | 3.04±0.89**     | 0.931 | 0.021 | 0.935      |
| Protein               | LC    | 2.90±0.35 | 3.26±0.16       | 3.10±0.67       | 3.18±0.10       |       |      |            |
|                       | HC    | 2.88±0.28 | 2.92±0.40       | 2.62±0.41*      | 2.68±0.22**     | 0.516 | 0.007 | 0.372      |
| Lactose               | LC    | 4.82±0.23 | 4.59±0.33       | 4.72±0.28       | 4.78±0.29       |       |      |            |
|                       | HC    | 4.90±0.28 | 4.96±0.09*      | 5.00±0.10       | 4.95±0.13*      | 0.845 | 0.017 | 0.646      |

LC, low concentrate; HC, high concentrate; Values are mean ± standard error of the mean (SEM), \( n=4 \) /group. \(^*p<0.05, **p<0.01 \) vs. LC.

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LPS content in ruminal fluid and plasma

LPS levels in ruminal fluids of HC group increased from 38,854 to 48,064 EU/mL before feeding in the morning and increased from 63,359 to 70,418 EU/mL after 8 h feeding compared to LC group. Plasma LPS concentration of HC goats was increased from 0.24 to 0.32 EU/mL before feeding in the morning and from 0.09 to 0.14 EU/mL after 4 h feeding compared to LC group. However, there was no significant difference in plasma LPS concentration and ruminal LPS content between HC and LC groups \( (p>0.05; \) Table 3).

Cytokines, acute phase proteins

Plasma concentrations of IL-6 and TNF-\( \alpha \) increased in HC group but were not significantly different compared to the LC group \( (p>0.05; \) Table 4). Compared with LC group, plasma SAA concentration in HC group was increased significantly \( (p<0.05) \), and plasma HP concentration also showed a tendency to increase \( (p=0.08) \). Results for plasma LBP were not significantly different for the HC group compared to the LC group \( (p=0.163) \).

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Table 2. The percentage of fat, protein, and lactose in milk from control and treatment group goats.

| Item                  | Diet  | SEM     | Effect, P-value |
|-----------------------|-------|---------|-----------------|
|                      |       |         | Diet  | Time | Diet × Time |
| Fat                   | LC    | 4.15±0.43 |       |      |            |
|                       | HC    | 3.09±0.63* |       |      |            |
| Protein               | LC    | 2.90±0.35 |       |      |            |
|                       | HC    | 2.88±0.28 |       |      |            |
| Lactose               | LC    | 4.82±0.23 |       |      |            |
|                       | HC    | 4.90±0.28 |       |      |            |

LC, low concentrate; HC, high concentrate; Values are mean ± standard error of the mean between two groups (SEM), \( n=4 \) /group. \(^*p<0.05, **p<0.01 \) vs. LC.

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Table 3. Lipopolysaccharide (LPS) levels in plasma and in rumen fluid of control and treatment group goats.

| Items                  | Diet  | SEM     | Effect, P-value |
|-----------------------|-------|---------|-----------------|
|                      |       |         | Diet  | Time | Diet × Time |
| Plasma LPS, EU/mL     | LC    | 0.24    | 0.32 | 0.05 | 0.249 | 0.002 | 0.716 |
|                       | HC    | 0.14    | 0.07 | 0.06 |       |      |      |
| Rumen LPS, EU/mL      | LC    | 38,854  | 48,064 | 20,383 | 0.717 | 0.084 | 0.98  |
|                       | HC    | 14,466  | 16,058 |       |       |      |      |

LC, low concentrate; HC, high concentrate; LPS, lipopolysaccharide; Values are mean ± standard error of the mean between two groups; \( n=4 \) /group. \(^*p<0.05, **p<0.01 \) vs. LC.

Statistical analysis conducted on log_{10}-transformed data.

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Table 4. Plasma cytokines and acute phase protein levels in control and treatment group goats.

| Parameters          | Diet  | HC     | P-value |
|---------------------|-------|--------|---------|
| IL-6(\mu g/mL)      | LC    | 221.32 | 345.45  |
|                     | HC    | 356.33 |        |
| TNF-\( \alpha \)(ng/mL) |   | 715.42 | 984.36  |
| SAA(\mu g/mL)       | LC    | 90.62  | 246.04  |
|                     | HC    | 130.46 |        |
| HP(\mu g/mL)        | LC    | 209.50 | 374.80  |
|                     | HC    | 130.46 |        |
| LBP(ng/mL)          | LC    | 23.32  | 34.48   |
|                     | HC    | 5.74   |        |

LC, low concentrate; HC, high concentrate; HP, haptoglobin; IL-6, interleukin-6; LBP, lipopolysaccharide binding protein; SAA, serum amyloid A protein; TNF-\( \alpha \), tumour necrosis factor \( \alpha \). Values are mean ± standard error of the mean (SEM), \( n=4 \) /group.

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The GeneRelNet (Co-expression network) analysis

Co-expression network analysis was used to find K-core regulatory factors (genes) (Figure 4A–D). For each pair of genes in a cluster, we calculated the Pearson correlation coefficient and chose the significant correlation pairs to construct the network (absolute value of interaction > 0.999). These genes were mainly involved in immune and inflammatory responses (n = 8), lipid metabolism (n = 5), protein metabolism (n = 26), carbohydrate metabolism (n = 7), out of a total of 153 differential expression genes (p < 0.01).

Real-time polymerase chain reaction (PCR) analysis involved in inflammatory response, glucose metabolism and lipid metabolism

The mRNA expression of hepatic TLR-4, IL-1β, SAA, CRP, and HP were significantly increased in HC group compared to LC counterparts (p < 0.05; Figure 5A). The expressions of hepatic TLR2, CD14, MyD88, TNF-α, and LBP did not show significant difference between HC and LC group (p > 0.05). Among these genes, only HP mRNA expression showed a more than 2-fold difference between the two groups, which was consistent with the data from the transcriptome microarray.

Expression of hepatic phosphoenolpyruvate carboxykinase (PC) mRNA was significantly down-regulated in HC group compared to LC group (p < 0.05) (Figure 5B). Hexokinase (HK) mRNA expression had a tendency to decrease in HC group (p = 0.07). However, other gluconeogenesis- and glycolysis-related genes transcription was not significantly altered in HC group compared to LC group (p > 0.05). The mRNA expression of hepatic acetyl-CoA acyltransferase 2 (ACAA2), acyl-CoA synthetase long-chain family member-1 (ACSL1), and hydroxyacyl-CoA dehydrogenase (HADH) genes were significantly increased (p < 0.05) in HC group compared to LC group (Figure 5C). There was a >2-fold difference in hepatic fatty acid desaturase 1 (FADS1) mRNA expression, but did not reach statistical significance due to the high variability between individual values. This result was consistent with the data from the microarray high-throughput data analysis.

Discussion

Dynamic changes in ruminal pH during SARA

Dairy ruminants are often fed high grain diets to meet the energy demand for high milk production. As a result, ruminal acidosis (especially SARA) often occurs during the periods of early and mid-lactation in dairy production herds [13] [11]. Due to the rapid fermentation and the accumulation of volatile fatty acid (VFA) in the rumen, as well as the increase of lactic acid, the value of rumen pH markedly decreased and prolonged for long time [28] [29]. In experimental study, rumen pH value has been considered as the most effective indicator of SARA. The thresholds of ruminal pH for the diagnosis of SARA have been set as ruminal pH < 5.6 [10] [30] [31] and < 5.8 [28] lasted for 3 hours per day using ruminal fluids detected by pH meter system in dairy cows [10]. Consistently, ruminal pH value < 5.8 lasted more than 3 hours was considered as the critical value of SARA diagnosis [32].

In this study, goats fed a high-concentrate diet for 2 weeks exhibited a significantly lower ruminal pH, which decreased to < 5.8 and persisted for more than 3 hours per day after feeding. According to the definition of experimental SARA, HC goats were suffered SARA disease. However, although ruminal pH value of HC goats remained significantly lower than LC goats from week 4 (4W) to week 8 (8W), the ruminal pH in HC goats was markedly lower.
Increased to more than 6.0. The increase in ruminal pH of HC goats indicated that the rumen was adapting to the high-concentrate diet, which was consistent with the study conducted in dairy cows by Steele et al [33].

Inflammatory response and HPA axis activation

It is widely accepted that during experimentally-induced SARA, the free LPS contents were increased in rumen fluids, yet LPS concentration in blood was vague after feeding HC diet that has been enriched with starch [15]. A decline in ruminal pH during SARA causes death and cell lysis of Gram-negative bacteria, the predominant bacterial group in the rumen, which results in an increase in free ruminal LPS concentration [12] [13,17]. Endotoxin produced in the digestive tract can be translocated into the bloodstream, which increases the concentration of blood LPS [5] [7]. It's generally accepted that LPS translocation into the bloodstream occurs as a consequence of grain-induced SARA, and that the intestines are likely the main site of LPS translocation [15] [16]. In grain-based SARA, more starch may flow into the hind gut especially into the large intestine where LPS may be produced in a significant amount as in the rumen via microorganism fermentation [9]. After LPS translocates into the peripheral circulatory system, the inflammatory and acute phase response will be activated [12]. Our results showed that, LPS concentrations were increased from 38,854 to 48,064 EU/mL before feeding in the morning and increased from 63,359 to 70,418 EU/mL after 8h feeding compared to LC group. Plasma LPS concentration of HC goats was increased from 0.24 to 0.32 EU/mL before feeding in the morning and from 0.09 to 0.14 EU/mL after 4 h feeding compared with LC group. However, due to the limited animal sizes and the individual variations the difference of LPS concentrations in ruminal fluids and plasma between HC and LC goats did not reach the statistical significance, it can’t be discarded whether this moderate increase of LPS in body fluids involved in activation of inflammatory response. The activation of TLR4 and IL-1β mRNA expression in liver of HC goats also supported the above speculation. Compared to LC group, HC goats showed a significant up-regulation in mRNA expression of APPs, including hepatic SAA, CRP, and HP mRNA, and paralleled a marked increase of SAA and HP in the plasma. These results were similar to results reported in dairy cattle [7] [13] [15].

The previous studies showed that the stress axis is activated when SARA is induced [34]. Cytokines and APPs are strong inducers that signal the HPA axis to release glucocorticoids from the adrenal gland [11] [35] [36]. In the present study, we found that goats fed a high-concentrate diet for 9 weeks had a significantly higher concentration of peripheral blood cortisol, which indicated that the HPA axis was activated during the long-term SARA challenge. This is consistent with limited, preliminary evidence that high concentrate diet (70% dry matter) adjustment to one-year-old Holstein heifers for 30 days increased blood total glucocorticoids from 7.9 to 14.8 ng/mL [37]. It's suggested that the increased concentration of glucocorticoids may be reflecting a metabolic adaptation to high concentrate feed [37]. In this study, hepatic GR protein expression was significantly down-regulated in liver of HC goats indicating a negative feedback from the higher level of plasma cortisol. As a primary and vital organ that is
involved in the immune response and in nutrient metabolism, the liver controls the input of precursors of milk components to the mammary gland [24] [38]. These changes of HPA axis may be aimed on redistribution of energy utilization in specific organs, inhibiting or stimulating energy mobilization according to priority [26].

The profile of mRNA expression in liver

In this study, microarray technology was used to investigate the hepatic gene expression profile and high-throughput data analysis was used to compare the differential expression of functional genes between HC and LC group. The Co-expression network of LC group shows in Figures S1, S2, S3, and S4. The activation of inflammatory and stress response in HC group was also confirmed from the GeneRelNet (Co-expression network) dataset. The results showed that the significant up-regulation (FC \(>2\), \(p<0.05\)) of key-core gene regulated genes included CD1 (NM_001123002) and CD4 (NM_001129902) molecules, IL-2 (X55641.1) and corticotropin releasing hormone binding protein (CRHBP, NM_001009339) in liver of HC goats. These genes are important for immune response, cell adhesion, and antigen presentation. It's well known that CD4 molecule plays a key role for IL-2 produce [39] and CD1 protein is important for the innate and adaptive immune response [40]. Moreover, the significant up-regulation of 3-hydroxybutyrate dehydrogenase 1 (BDH1, NM_001034600.1) and Dipeptidase 1 (DPEP1, NM_001034472.1), and the down-regulation of glutathione peroxidase 2 (GPX2, NM_001163139.1) and aldehyde dehydrogenase 18A1 (ALDH18A1, NM_001046181.1) indicated the decrease of anti-oxidative capacity in liver of goat feeding high concentrate diet [41] [42] [43]. In a good agreement, feeding high concentrate diets enrich starch to dairy cow induced SARA disease and led to systemic inflammatory response due to the translocation of LPS from gastrointestinal tract into the peripheral circulation [10] [41] [42].

In addition to being an energy source, protein plays a functional role in many organs including the liver. The changes in protein metabolism are critical to maintaining cell size and directly impacts cell survive. Gene ontology analysis also identified 26 differential expression genes related to protein metabolism. Majority of these genes are involved in the biological processes of proteolysis, collagen catabolism, and purine nucleotide metabolism. Among these genes, two ATPase (NM_001083653.1,
XM_001788345.1), branched chain amino-acid transaminase 1 (BCAT1, NM_001083644.1) and fascin homolog 3 (FSCN3, NM_001075543.2) were significantly up-regulated in HC goats, while RNA polymerase III (NM_001083750.1), and three kinases (XM_866451.4, NM_001109962.1, NM_001078008.1) were markedly down-regulated. These changes likely indicated the priority of protein catabolism in liver of HC goats compared to LC. It’s reported that the elevation of plasma cortisol levels inhibits protein synthesis while increasing protein breakdown [43]. In this study, the relationship between increased plasma cortisol level and protein breakdown still warrants further investigation.

The priority of catabolism was also observed in lipids metabolism. Our results showed that the transcription of genes encoding three key enzymes acting in fatty acids β-oxidation [44], ACAA2, ACSL1 and HADH were up-regulated in liver of HC goats, which was consistent with the increase of NEFA level in blood. As a metabolic hormone, leptin plays vital roles on food intake, energy expenditure, and body weight mediated by leptin receptors situated in the hypothalamus [45]. Leptin has also been shown to have effects on whole body glucose and lipid metabolism [46]. In this study, the significant increase of leptin in HC goats paralleled the increase of NEFA, which indicates the stimulatory effect of leptin on lipids catabolism. With respect to glucose metabolism, key-core gene glycogen synthase (GYS2, NM_001192905.1) mRNA transcription was down-regulated in HC goats. In addition, Q-PCR results showed that mRNA expression of two important gluconeogenic genes hexokinase (HK) and pyruvate carboxylase (PC) genes was significantly down-regulated in liver of HC group. These data suggests the decrease of glucogen synthesis and gluconeogenesis in liver of HC group. However, this is contradictory to the increase of blood glucose concentrations, which was significantly elevated in HC group. This increase of blood glucose might be related to the production of lactose in these lactating animals [47]. Previous study showed that fatty acids have shown to produce an insulin-resistant state via cascade signaling pathway involving protein kinase C (PKC) and AKT phospholation [48]. Also, high levels of free fatty acids in the plasma have been shown to induce insulin resistance [49]. However, whether the increase of blood glucose in HC group is due to insulin-resistance is still unclear.

### Table 5. Nutrient composition and forge to concentrate ratio (F:C) of the TMR.

| Items                | LC   | HC   |
|----------------------|------|------|
| Leymus chinensis     | 40   | 26.7 |
| Alfalfa silage       | 20   | 13.3 |
| Corn                 | 22.99| 23.24|
| Bran                 | 0    | 20.77|
| Soybean meal         | 15   | 13.66|
| Limestone            | 0.65 | 1.43 |
| CaHPO₄               | 0.46 | 0    |
| Premix               | 0.5  | 0.5  |
| NaCl                 | 0.4  | 0.4  |
| Total                | 100  | 100  |
| ME (MJ/kg)           | 5.63 | 5.83 |
| DCP %                | 9.9  | 10   |
| NDF %                | 36.64| 34.55|
| ADF %                | 24.74| 20.35|
| EE %                 | 2.87 | 3.21 |
| NFC %                | 31.76| 35   |
| Ca %                 | 0.8  | 0.9  |
| P %                  | 0.33 | 0.38 |
| H2O%                 | 88.4 | 88.1 |

*Amount of premix added, VA: 6000 IU/kg, VD: 2500 IU/kg, VE: 80 mg/kg, Cu: 6.25 mg/kg, Fe: 62.5 mg/kg, Zn: 62.5 mg/kg, Mn: 50 mg/kg, t: 0.25 mg/kg, Se: 0.125 mg/kg, Co: 0.125 mg/kg, Mo: 0.125 mg/kg.

Nutrient levels were calculated values.

LC, low concentrate; HC, high concentrate.
### Table 6. PCR primers for immunity and stress, lipid metabolism and carbohydrate metabolism.

| Target genes | Genbank accession | PCR products (bp) | Primer sequences |
|--------------|-------------------|------------------|-----------------|
| β-actin      | AF_481159         | 260              | F:5'-CGGGATCCATCTGCGTGACCT-3'<br>R:5'-GGGATCCGAGGAGCTTG-3' |
| ACAA2        | XM_004020663.1    | 165              | F:5'-TGCTGCTGCAAATGACCT-3'<br>R:5'-AACCCAGACGACGACGACG-3' |
| HADH         | XM_004009637.1    | 197              | F:5'-GAGTTTGTGGCAGACTCT-3'<br>R:5'-GGGCTTGTATCAGGAGGAGAAG-3' |
| CPT1         | Y18830.1          | 158              | F:5'-CTTGTGACACTTTGTGC-3'<br>R:5'-CCTTAAGCAGTTGTTGCG-3' |
| CPT2         | BC105423.1        | 157              | F:5'-CTTGTGACACTTTGTGC-3'<br>R:5'-CCTTAAGCAGTTGTTGCG-3' |
| ACSL1        | XM_001076085.1    | 191              | F:5'-GCAHCCCAAGGCAATG-3'<br>R:5'-AGCAGCTGTATCCCT-3' |
| PPARα        | HM600810.1        | 243              | F:5'-TTAAGGCAACCAAGATAACC-3'<br>R:5'-TCACCAAAAGCCGGAAGA-3' |
| ATGL         | GQ918145.1        | 180              | F:5'-GGAGCTTATCCAGGC-3'<br>R:5'-TGCGGGCAGATGTCACTC-3' |
| ACSS1        | BC055008.1        | 242              | F:5'-TCCTTCTGGGGAGGATCAA-3'<br>R:5'-CTTCTGATGAGGTAC-3' |
| LPL          | JQ670882.1        | 235              | F:5'-TTAAGGCAACCAAGATAACC-3'<br>R:5'-TCACCAAAAGCCGGAAGA-3' |
| SCD          | AF325499.1        | 178              | F:5'-TGCTGACACTTTGTGC-3'<br>R:5'-CCTTAAGCAGTTGTTGCG-3' |
| DGAT1        | DQ380249.1        | 240              | F:5'-TGCCCTAGACACTTCAAGG-3'<br>R:5'-GCCGATTGAGTGCAGACG-3' |
| DGAT2        | AUS19787.1        | 234              | F:5'-CACTGCTGAGCCTACTCTCA-3'<br>R:5'-TTTCTGGTGTCTCCAGTCA-3' |
| FADS1        | XM_004019593.1    | 187              | F:5'-CCTTGTGACACTTATCTAC-3'<br>R:5'-ACAAACCAGTTGCTTCCAGG-3' |
| FADS2        | AY.850395.1       | 257              | F:5'-AGGCCCTGCTAGCCTACTAC-3'<br>R:5'-CCTTGTGGAGCATTGGGT-3' |
| ACACA        | NM_001009256      | 230              | F:5'-CATGCCAATGCTACGGG-3'<br>R:5'-GGTGGTAGATGGGAGAGGA-3' |
| FAS          | XM_004013447.1    | 112              | F:5'-TGCTGATGACACTTCTC-3'<br>R:5'-AGGTATGCCCCCTTCCG-3' |
| PPARγ        | JN854246.1        | 238              | F:5'-CATTCTTGCTCCGAGC-3'<br>R:5'-TGGAAACTCATGTTCTC-3' |
| PC           | NM_177946         | 187              | F:5'-TGGGCTTGTGACACTC-3'<br>R:5'-AGGCTTTTTAAGGCAGAGAGG-3' |
| FBP1         | NM_001034447      | 106              | F:5'-GGGTAAAGCCATCTC-3'<br>R:5'-CATCCAGCTTCTTACACTTTCC-3' |
| G6Pase        | EF062861          | 158              | F:5'-AATGCTATGTTGTGGAT-3'<br>R:5'-GAATTCATGTTGTGGGAT-3' |
| HK1          | AM492192          | 139              | F:5'-CGCCGCTTCTGAGAACTCT-3'<br>R:5'-TGAGCCATCGGAAATAGA-3' |
| PYGL         | AY827551          | 150              | F:5'-GCTCCCAAGTAATGGT-3'<br>R:5'-GGGATCCGAGGAGCTTG-3' |
| LEPR         | NM_001009763      | 110              | F:5'-GGGATCCGAGGAGCTTG-3'<br>R:5'-CTTGAAGAGATTATATTGGGAC-3' |
| GYS2         | NM_001192905      | 131              | F:5'-TGCCGGAGATGTTTTGG-3' |
Taken as a whole, our data suggest that feeding HC diet to lactating goats for long period caused the activation of the inflammatory and stress response, and altered substrates metabolism in liver. Based on the data presented in this study, limited conclusions can be drawn from just measuring mRNA abundance because the post-transcription, protein translation, and the amount of active protein are dependent on many factors [50]. To our knowledge, it’s the first time to report the changes of metabolism in liver after feeding HC diet to lactating ruminants, and the determination of wide-genome expression in liver will contribute to find the specific biochemical pathways.

Conclusion
In summary, feeding a high-concentrate diet to lactating goats for the long-term will result in the depression of milk fat and milk protein. Activation of the stress response, the acute phase response stimulation and the inflammatory response may contribute to nutrient partitioning and re-distribution of energy in the liver, and ultimately lead to a decline in milk quality.

Materials and Methods

Ethics Statement
The experiment was conducted following the guidelines of Animal Ethics Committee at Nanjing Agricultural University, China. The study was approved by Animal Ethics of Nanjing Agriculture University. The sampling procedures complied with the “Guidelines on Ethical Treatment of Experimental Animals” (2006) No. 398 set 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.
Animals
Eight health multiparous mid-locating goats with average initial BW 47 ± 8 kg (mean ± SD) were housed in individual stalls in a standard animal feeding house at Nanjing Agricultural University (Nanjing, China). Animals were cannulated in rumen and were randomly allocated to two groups, one receiving diets with low concentrate (40% of dry matter) as the LC group (n = 4), and another receiving high concentrate diet (60% of dry matter) as the HC group (n = 4) for 9 weeks. Ingredient and nutrient composition of experimental diets were presented in Table 3. Goats were free access to fresh water throughout the experimental time.

Milk composition analysis
Fifty milliliter of milk were sampled every two weeks into the vials with potassium dichromate at 8:00 in the morning, and stored at the 4°C until milk fat, protein and lactose analysis conducted by the commercial company of Nanjing Weigang dairy industry Co., Ltd. The data were analyzed by Univariate using the General Linear Models of SPSS 11.0 for Windows (StatSoft Inc, Tulsa, OK, USA).

Rumen fluid collecting and measurements
Fifteen minutes prior to feed delivery and 1, 2, 3 and 5 h (or 1, 3, 5 h) after feed delivery on 2 consecutive days during 2, 4, 6 and 8 week of experimental period, 15 mL rumen fluids was collected with nylon bag and divided into 5 mL for pH measurement immediately with pH-meter. Another 10 mL were transferred into tubes and kept on ice and then centrifuged at 1,900 × g at 4°C for 30 min. After that, the supernatant was collected and stored at −20°C until for LPS analysis. The concentration of LPS in rumen fluid was determined by a chromogenic endpoint assay (CE64406, Chinese Horseshoe Crab Reagent Manufacturer Co., Ltd., Xiamen, China) with a minimum detection limit of 0.1 EU/mL. The inter- and intra- assay coefficients of variation were 5% and 10%, respectively. The concentration of NEFA (A042) and glucose was detected by commercial kits purchased from Shanghai Institute of Biological Products. The ranges of assay sensitivity were between 0.5 and 24 ng/mL. The inter- and intra- assay coefficients of variation were 9% and 10% respectively.

Measurement of plasma biochemical parameters
At the end of experiment, plasma was sampled fifteen minutes prior to feed delivery using EDTA-containing vacuum tubes from jugular vein. Plasma leptin concentration was measured with radioimmunnoassay (RIA) using commercial kits purchased from Shanghai Institute of Biological Products. The ranges of assay sensitivity were between 0.5 and 24 ng/mL. The inter- and intra- assay coefficients of variation were 5% and 10%, respectively. The concentration of NEFA (A042) and glucose was detected by commercial kits purchased from Nanjing Jiancheng Bioengineering institution, and the procedures were performed according to the manufacture’s instruction. The concentrations of leptin and glucose were carried out in Nanjing General Hospital of Nanjing Military. Inflammatory cytokines such as IL-6 (RGB-60023G, Beijing Rigorbio Science Development Co., Ltd.), TNFα (RGB-60080G) and acute phase protein such as LBP (RGB-60266G), HP (H086-09, abcam), SAA (ab100635, abcam) were detected by ELISA kits. The concentration of LPS in plasma was determined by a chromogenic endpoint assay with diazo coupling reagent (CE80545, Chinese Horseshoe Crab Reagent Manufacturer Co., Ltd., Xiamen, China) with a minimum detection limit of 0.01 EU/mL. The procedures were performed according to the manufacture’s instruction. The data were analyzed by Independent-Samples T test using the Compare Means of SPSS 11.0 for Windows (StatSoft Inc, Tulsa, OK, USA).

RNA extraction and real-time quantitative PCR
The liver tissues were sampled by a punch biopsy with a local anesthesia. Total RNA was extracted from each dairy goat using TRIZOL reagent (Takara, Dalian, China) according to the manufacturer’s protocols. Total RNA concentration was then quantified by measuring the absorbance at 260 nm in a spectrophotometer (Eppendorf Biotechnology, Hamburg, Germany). Aliquots of RNA samples were subjected to electrophoresis with 1.4% agarose-formaldehyde gels stained with ethidium bromide to verify their integrity. Reverse transcription (RT) was performed using the total RNA (2 μg) in a final volume of 25 μL containing 1 × RT-buffer, 100 U reverse transcriptase, 8 U RNase inhibitor (Promega, USA), 5.3 μmol/L random hexamer primers and 0.8 nmol/L dNTP (TaKaRa, Dalian, China). After incubation at 37°C for 1 h, the reaction was terminated by heating at 95°C for 5 min and quickly cooling on ice. Real-time PCR was performed in Mx3000P (Stratagene, La Jolla, CA, USA). Mock RT and No Template Controls (NTC) were set to monitor the possible contamination of genomic DNA both at RT and PCR. Melting curves were performed to insure a single specific PCR product for each gene. Two microliter of 16-fold dilution of RT product was used for PCR in a final volume of 25 μL containing 12.5 μL SYBR Green Real-time PCR Master Mix (TOYOBO Ltd., Shanghai, China) and 0.6–0.8 μM of each forward and reverse primers for target genes (as shown in Table 6). Goat β-actin mRNA was used as a reference gene for normalization purposes. The following PCR protocols were initial denaturation (1 min at 95°C), then a three-step amplification program (20 s at 95°C, 20–30 s at 60–64°C, 30 s at 72°C) was repeated for 45 times. The method of 2−ΔΔCt was used to analyze qPCR data with Independent-Samples T test using Compare means of SPSS 11.0 for Windows to statistical analysis. All samples were included in the same run of RT-PCR and repeated for at least 3 times. The primers were shown in Table 6. The data was analyzed by Independent-Samples T test using the Compare Means of SPASS 11.0 for Windows (StatSoft Inc, Tulsa, OK, USA).

Western blotting
Total protein was extracted from frozen liver tissue, and the protein concentration was determined by the BCA assay (Pierce, Rockford, IL, USA). Fifty micrograms of protein extract from each sample were subjected to electrophoresis on a 10% SDS-PAGE gel, and the separated proteins were transferred onto the nitrocellulose membranes (Bio Trace, Pall Co., USA). Western blot analysis for GR (sc-1004, Santa Cruz Biotechnology, 1:500) is performed in Mx3000P (Stratagene, La Jolla, CA, USA). Mock RT and No Template Controls (NTC) were set to monitor the possible contamination of genomic DNA both at RT and PCR. Melting curves were performed to ensure a single specific PCR product for each gene. Two microliter of 16-fold dilution of RT product was used for PCR in a final volume of 25 μL containing 12.5 μL SYBR Green Real-time PCR Master Mix (TOYOBO Ltd., Shanghai, China) and 0.6–0.8 μM of each forward and reverse primers for target genes (as shown in Table 6). Goat β-actin mRNA was used as a reference gene for normalization purposes in the Western blot analysis. Finally, the blot was washed and detected by enhanced chemiciluminescence (ECL) using the Lumihlo substrate (Super Signal West Pico Trial Kit, Pierce, USA). ECL signals were recorded by an imaging system (Bio-Rad, USA) and analyzed with Quantity One software (Bio-Rad, USA). Values of GR protein was presented as fold change relative to the average value of LC group. The data was analyzed by Independent-Samples T test using the Compare Means of SPASS 11.0 for Windows (StatSoft Inc, Tulsa, OK, USA).

mRNA Microarray Experiment
Transcriptome microarrays were performed for seven dairy goats (3 goats from the LC group and 4 goats from the HC group) for hepatic gene expression profiling. Because complete genome sequence data was not available, gene sequences from three
animal species were employed to design the gene chip (goat, sheep, and Bos Taurus). Goat expressed sequence tag (EST) sequence (n = 13990), goat gene sequence (n = 720), sheep gene sequence (n = 2718), and Bos Taurus gene sequence (n = 35000) transcripts were investigated using the microarray. There were 61012 probe IDs and 2-fold difference probe IDs (n = 2140). Reference sequences were cited from http://www.ncbi.nlm.nih.gov/nucest/. The FDR (False Discovery Rate) were calculated. Differentially expressed genes (DEGs) were selected with FDR <5% and FDR <10%. All data were MIAME compliant and have been deposited in GEO (accession number GPL17623 and GPL17624).

GO analysis

GO analysis was applied to analyze the main function of the differential expression genes according to the Gene Ontology which is the key functional classification of NCBI, which can organize genes into hierarchical categories and uncover the gene regulatory network on the basis of biological process and molecular function [51]. Specifically, two-side Fisher’s exact test and $\chi^2$ test were used to classify the GO category, and the false discovery rate (FDR) [32] was calculated to correct the P-value; the smaller the FDR, the smaller the error in judging the p-value. The FDR was defined as $FDR = 1 - \frac{N_f}{T}$, where $N_f$ refers to the number of Fisher’s test P-values less than $\chi^2$ test P-values. We computed P-values for the GOs of all the differential genes. Enrichment provides a measure of the significance of the function: as the enrichment increases, the corresponding function is more specific, which helps us to find those GOs with more concrete function description in the experiment. Within the significant category, the enrichment Re was given by: $Re = (n_f/n)/(N_f/N)$ where “n” is the number of flagged genes within the particular category, “n” is the total number of genes within the same category, “Nf” is the number of flagged genes in the entire microarray, and “N” is the total number of genes in the microarray [53].

Supporting Information

Figure S1 Co-expression network for immune and inflammatory responses in the liver of LC goats. (TIF)

Figure S2 Co-expression network for lipid metabolism in the liver of LC goats. (TIF)

Figure S3 Co-expression network for protein (or amino acid) metabolism in the liver of LC goats. (TIF)

Figure S4 Co-expression network for carbohydrate metabolism in the liver of LC goats. (TIF)

Author Contributions

Conceived and designed the experiments: YN YZ SZ XS. Performed the experiments: HD YW. Contributed reagents/materials/analysis tools: RZ. Wrote the paper: HD YN.

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