Effects of Dietary Nitrogen Level on Urinary Purine Derivative Excretion and Rumen Microbial Nitrogen Production in Yaks (Postprint)

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Abstract

This study was conducted to investigate the response pattern of purine derivative (PD) excretion in yak urine to dietary nitrogen levels, and to estimate rumen microbial nitrogen (MN) production based on this relationship, thereby providing reference for scientific feeding practices for yaks in alpine pastoral regions. Four castrated male yaks with similar body weight [(192$\pm$12)*kg*]*andidenticalage*(3*years*)*wereselectedandallocatedto*4*groupsusinga*4×$4 Latin square design, with dietary nitrogen levels of 1.03%, 1.95%, 2.85%, and 3.76%, respectively, one yak per group. The experiment comprised 4 periods, each lasting 21 days, consisting of a 15-day preliminary period followed by a 6-day formal collection period. The results indicated that PD in yak urine was primarily composed of allantoin and uric acid, with allantoin/PD and uric acid/PD ratios of 0.69~0.76 and 0.23~0.30, respectively, while xanthine and hypoxanthine contents were negligible. As dietary nitrogen level increased, urinary excretion of PD, allantoin, uric acid, and hippuric acid increased linearly (P<0.05), whereas uric acid/PD and purine nitrogen index (PNI) decreased linearly (P<0.05). Rumen bacterial purine base (RNA equivalent) content, rumen bacterial nitrogen content, and rumen MN production all increased linearly with increasing dietary nitrogen level (P<0.05); however, the efficiency of dietary nitrogen utilization for MN synthesis [i.e., rumen MN/nitrogen intake (NI)] decreased linearly (P<0.05). Based on the strong linear relationships between urinary PD excretion (mmol/d) and rumen MN production (g/d) with NI (g/d), the following mathematical models were established: PD=0.58NI+18.28, MN=0.18NI+22.18. In conclusion, when dietary nitrogen level was 2.85%, rumen MN production reached its maximum value of 42.60 g/d, while PNI and the efficiency of dietary nitrogen utilization for MN synthesis were highest under low nitrogen (1.03%) conditions. These results reveal the characteristic of efficient nitrogen utilization from low-nitrogen diets

in yaks and elucidate the nutritional mechanism underlying yak adaptation to nutrient-deficient forage on the Qinghai-Tibet Plateau.

Full Text

Effects of Dietary Nitrogen Level on Urine Purine Derivatives Excretion and Microbial Nitrogen Production in Yaks

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Abstract: This experiment was conducted to investigate the response patterns of urinary purine derivatives (PD) excretion to dietary nitrogen levels in yaks and to estimate ruminal microbial nitrogen (MN) production based on these patterns, thereby providing a reference for scientific feeding of yaks in alpine pastoral regions. Four castrated male yaks of similar body weight $[(192\$\pm12)kg]andage(3years)wereselectedanddividedintofourgroupsusinga4\times\$4$ Latin square design. The dietary nitrogen levels for each group were 1.03%, 1.95%, 2.85%, and 3.76%, respectively, with one yak per group. The experiment consisted of four periods, each lasting 21 days (including a 15-day preliminary period and a 6-day formal collection period). The results showed that urinary PD in yaks were primarily composed of allantoin and uric acid, with allantoin/PD and uric acid/PD ratios of 0.69–0.76 and 0.23–0.30, respectively, while xanthine and hypoxanthine contents were negligible. As dietary nitrogen level increased, urinary excretion of PD, allantoin, uric acid, and hippuric acid increased linearly ($P<0.05$), whereas uric acid/PD and purine nitrogen index (PNI) decreased linearly ($P<0.05$). Ruminal bacterial purine bases (RNA equivalent) content, bacterial nitrogen content, and MN production all increased linearly with dietary nitrogen level ($P<0.05$), but the efficiency of dietary nitrogen utilization for MN synthesis [i.e., ruminal MN/nitrogen intake (NI)] decreased linearly ($P<0.05$). Based on the strong linear relationships between urinary PD excretion (mmol/d), ruminal MN production (g/d), and NI (g/d), the following mathematical models were established: PD=0.58NI+18.28 and MN=0.18NI+22.18. In conclusion, ruminal MN production reached its maximum (42.60 g/d) when dietary nitrogen level was 2.85%, while PNI and the efficiency of dietary nitrogen conversion to MN were highest under low nitrogen conditions (1.03%). These results reveal the characteristic of efficient nitrogen utilization from low-nitrogen diets in yaks and explain the nutritional mechanism underlying their adaptation to the nutrient-deficient forage of the

Qinghai-Tibetan Plateau.

Keywords: yak; dietary nitrogen level; urinary purine derivatives; ruminal microbial nitrogen production

Introduction

Compared with monogastric animals, ruminants are characterized by possessing a large-volume rumen that harbors an astonishing quantity of microorganisms. Rumen microorganisms not only secrete large amounts of digestive enzymes to help the host degrade fibrous feed but also provide various amino acid resources required for nutritional metabolism. It has been reported that more than half of the amino acids absorbed by ruminants in the small intestine originate from rumen microorganisms [**?**], and under nutritional stress conditions, rumen microorganisms are virtually the only digestible protein source for the host [**?**]. Therefore, accurate quantification of ruminal microbial nitrogen (MN) production is of great significance for evaluating nitrogen utilization efficiency in ruminants.

Ruminal MN production is typically estimated using markers. In early studies, markers mainly included isotopes ($^{15}NH_3$, $^{35}SO_4{}^{2-}$, etc.), 2,6-diaminopimelic acid, D-alanine, nucleic acids (DNA or RNA), and purines. However, these methods require the use of fistulated animals, which not only increases experimental costs and limits animal numbers but also raises animal welfare concerns [**?**]. The urinary purine derivatives (PD) method can overcome these disadvantages as it is simple to operate and highly accurate, thus gaining rapid development and application [**?**]. Since the 1980s–1990s, application models for this method have been successively established in various ruminants including sheep [**?**], goats [**?**], cattle [**?**], buffalo [**?**], zebu [**?**], and yaks (*Bos grunniens*) [**?**].

The yak is a unique livestock species on the Qinghai-Tibetan Plateau, possessing strong adaptability to harsh environmental conditions such as high altitude, cold, hypoxia, strong ultraviolet radiation, and short grass-growing seasons [**?**]. Through centuries of natural and artificial selection, yaks may have developed a series of special nutritional metabolism mechanisms to resist the threat of insufficient forage supply during the cold season, thereby ensuring normal reproduction and survival of the population [**?**]. Studies have shown that yaks have low nitrogen maintenance requirements [0.40–0.53 g/(kg $W^{0 \cdot 75} \cdot$ d)] [**?**], higher dietary nitrogen utilization efficiency than Holstein cows [**?**], and under nitrogen stress conditions, 87% of urea synthesized by the yak liver can be recycled into the digestive tract to provide nitrogen sources for rumen microbial protein synthesis [**?**]. These findings collectively suggest that yak microbial protein synthesis efficiency may be higher than that of other low-altitude cattle breeds. Therefore, this study aimed to investigate the effects of dietary nitrogen level on urinary PD excretion patterns in yaks and to estimate ruminal

MN production, thereby providing a theoretical basis for revealing the special nitrogen nutritional metabolism mechanisms of yaks.

1.1 Experimental Location and Duration

The feeding trial and sample collection were conducted from November 2013 to January 2014 at the Wushaoling Yak Experimental Station in Tianzhu Tibetan Autonomous County, Gansu Province (37°12.479 N, 102°51.695 E, altitude 3,154 m). Indoor sample analysis was performed from February to May 2014 at the International Center for Tibetan Plateau Ecosystem Management, Lanzhou University.

1.2 Experimental Animals and Diets

Four healthy castrated male yaks of similar body weight [(192$±12)*kg*]*andage*(3*years*)*wereselected.Beforethee* *dayadaptationperiodtofamiliarizethemwiththefeedingconditions*, *experimentalpersonnel*, *andsurrounding* *to*−*forageratiowas*50 : 50, *withhighlandbarleystrawastheroughageandfourtypesofpelletedconcentrateswith* d)] [**?**].

1.3 Experimental Design and Management

This experiment employed a 4$×$4 Latin square design. The entire trial consisted of four periods, each lasting 21 days (including a 15-day preliminary period and a 6-day formal collection period). The experimental yaks were fed twice daily at 08:00 and 18:00, with 1.5 kg dry matter per feeding. Water was provided ad libitum, and body weight was measured on an empty stomach on the first and last day of each period.

1.4 Sample Collection and Processing

On the first day of the formal collection period in each period, total urine collection was conducted continuously for 5 days before morning feeding, and daily urine volume per yak was recorded. Daily urine samples were mixed thoroughly, and 10% of the total volume was sampled and acidified with 50% H_2SO_4 to pH<3.0 to fix urinary nitrogen and prevent microbial growth, then stored at -20°C. On the last day of the formal collection period, rumen fluid was collected using an oral stomach tube before morning feeding and at 2, 4, 6, and 8 h post-feeding, with approximately 100 mL collected each time. The samples were filtered through four layers of gauze, placed in centrifuge tubes, and stored at -20°C.

1.5 Measurement Methods and Calculation Formulas

Urinary PD contents (allantoin, uric acid, xanthine, and hypoxanthine) in yaks were determined using high-performance liquid chromatography (Agilent LC-1200) following the method of Li et al. [**?**]. Rumen microbial extraction followed the method of Wickersham et al. [**?**]. Rumen bacterial purine nitrogen (PN)

content was measured according to Zinn et al. [**?**], and rumen bacterial nitrogen (BN) content and urinary nitrogen excretion were determined using the Kjeldahl method [**?**].

Ruminal MN production in yaks was calculated based on previously established [**?**] and modified models [**?**]. The relationship between purine absorption in the small intestine (X, mmol/d) and urinary PD excretion (Y, mmol/d) is as follows:

$$Y = 0.85X + 0.134BW^{0.75}$$

where $BW^{0.75}$ is metabolic body weight (kg).

The formula for estimating ruminal MN production is:

$$MN(g/d) = \frac{X \times 70}{(PN : BN) \times 0.83 \times 1000}$$

where X is purine absorption in the small intestine (mmol/d); 70 refers to the nitrogen content per millimole of purine (70 mg); 0.83 refers to the digestibility of microbial nucleic acids.

Purine nitrogen index (PNI) = urinary purine nitrogen excretion / urinary nitrogen excretion [**?**].

1.6 Data Processing and Analysis

Experimental data were analyzed for significant differences using polynomial orthogonal contrasts with the SAS 9.2 PROC MIXED module, with dietary nitrogen level as a fixed factor and experimental animal and period as random factors. Linear correlation analysis and model relationship plots were performed using R Studio software.

Results

2.1 Urinary PD Excretion and Small Intestine Purine Absorption

As shown in Table 2 , urinary PD in yaks were primarily composed of allantoin and uric acid, with xanthine and hypoxanthine contents being negligible and unaffected by dietary nitrogen level ($P>0.05$). With increasing dietary nitrogen level, urinary excretion of allantoin, uric acid, and PD, as well as small intestine purine absorption, increased linearly ($P<0.05$). The allantoin/PD ratio ranged from 0.69 to 0.76, while the uric acid/PD ratio ranged from 0.23 to 0.30. The allantoin/PD ratio increased linearly with dietary nitrogen level ($P<0.05$), whereas the uric acid/PD ratio showed the opposite trend. Urinary hippuric acid excretion increased linearly with dietary nitrogen level ($P<0.05$), while PNI decreased linearly ($P<0.05$), ranging from 0.06 to 0.22 and reaching its maximum at the 1.03% dietary nitrogen level.

2.2 Rumen Bacterial Parameters and MN Production

As shown in Table 3 , rumen bacterial purine bases (RNA equivalent), rumen bacterial PN, rumen bacterial BN content, and the rumen bacterial PN/rumen bacterial BN ratio all increased linearly with dietary nitrogen level (P<0.05), with the rumen bacterial PN/rumen bacterial BN ratio ranging from 0.12 to 0.20. Ruminal MN production also increased initially and then decreased with increasing dietary nitrogen level, reaching its maximum of 42.60 g/d at the 2.85% dietary nitrogen level. The ruminal MN/NI ratio decreased linearly with dietary nitrogen level (P<0.05), indicating that the efficiency of dietary nitrogen conversion to MN gradually decreased.

Due to the high linear correlation between nitrogen intake (NI, g/d) and both PD (mmol/d) and MN (g/d), mathematical models were established through linear regression analysis (Figure 1 [Figure 1: see original paper]):

$$PD = 0.58NI + 18.28 \quad (R^2 = 0.93)$$
$$MN = 0.18NI + 22.18 \quad (R^2 = 0.60)$$

Discussion

3.1 Response Pattern of Urinary PD Excretion to Dietary Nitrogen Level in Yaks

Urinary PD in ruminants primarily originate from rumen microbial nucleic acids [**?**], and ruminal MN production increases with dietary nitrogen level, leading to increased urinary excretion of PD and their components. This is consistent with the findings of Guo et al. [**?**] in yaks and Zhou [**?**] in sheep. In this experiment, small intestine purine absorption increased linearly with dietary nitrogen level because elevated nitrogen intake promoted the anabolic metabolism of microbial nucleic acids in the rumen [**?**], and when microbial nucleic acids were degraded to purines, purine absorption by the small intestine mucosa consequently increased.

Urinary PD in ruminants consist of allantoin, uric acid, xanthine, and hypoxanthine, with allantoin and uric acid comprising the major proportion [**?**], while xanthine and hypoxanthine constitute a minor proportion [**?**]. The present results showed that allantoin/PD and uric acid/PD ratios in yaks were 0.69–0.76 and 0.23–0.30, respectively, consistent with results reported by Long et al. [**?**] and Wang et al. [**?**] in yaks, and similar to those reported by Chen et al. [**?**] for cattle. With increasing dietary nitrogen level, allantoin/PD increased linearly while uric acid/PD decreased linearly, showing the same trend as reported by Long et al. [**?**] and Wang [**?**]. The results indicated that xanthine and hypoxanthine contents in yak urine were very low or absent (<1%), similar to findings in cattle reported by Chen [**?**], but relatively higher in sheep (16.1%). This difference may be related to xanthine oxidase activity in animals. Compared with sheep, bovine species have higher xanthine oxidase activity in liver, blood, and

small intestine mucosal cells, making xanthine and hypoxanthine easily oxidized to uric acid [?, ?].

3.2 Response Pattern of Rumen Bacterial Purine Bases, BN Content, MN Production, and PNI to Dietary Nitrogen Level

In this experiment, rumen bacterial purine bases (RNA equivalent) content in yaks ranged from 5.42% to 12.53%, with an average of 8.97%, similar to results reported by Han et al. [?] in yaks (8.3%–11.4%, average 9.85%) but higher than those reported by Smith et al. [?] in dairy cows and sheep (5.2%–6.8%, average 5.7%). BN content (4.28%–6.21%) was also similar to results from Han et al. [?] (4.64%–5.92%) but lower than that in dairy cows (5.76%–9.12%) [?]. Zhou [?] also found in a study on nitrogen stress adaptation in Tibetan sheep that rumen microbial RNA content (6.62%–8.17%) was higher than that in fine-wool sheep, while BN content (2.96%–3.18%) was relatively lower. Therefore, rumen microorganisms in high-altitude ruminants (such as Tibetan sheep and yaks) are characterized by high RNA and low nitrogen content, which may be a result of long-term adaptation to the nutritional stress of forage on the Qinghai-Tibetan Plateau. In early studies, the rumen bacterial PN/rumen bacterial BN ratio was considered relatively stable, with an average value of 0.116 [?]. However, further research revealed that this ratio varies considerably and is influenced by factors such as feed intake, microbial community composition, and animal breed [?]. In this experiment, the rumen bacterial PN/rumen bacterial BN ratio fluctuated between 0.12 and 0.20, indicating that dietary nitrogen level also affects this ratio.

Ruminal MN production increased linearly with nitrogen level, consistent with trends reported by Sannes et al. [?] and Devant et al. [?]. At the 1.95% dietary nitrogen level, ruminal MN production was 33.56 g/d, similar to the 31.1 g/d reported by Guo et al. [?] at the 1.97% dietary nitrogen level. The ruminal MN/NI ratio reflects the efficiency of dietary nitrogen conversion to MN by rumen microorganisms. In this experiment, this ratio reached its maximum (0.82) under low nitrogen conditions (1.03% dietary nitrogen), indicating high efficiency of dietary nitrogen conversion to MN under low-nitrogen diets. This also confirms that under nitrogen nutritional stress, microbial protein is the most important amino acid source for the host. The lowest ruminal MN/NI ratio (0.34) was observed under high nitrogen conditions (3.76% dietary nitrogen), because when yaks consume high-nitrogen diets, the amount of rumen-undegradable protein entering the small intestine increases, reducing the host' s dependence on amino acids provided by microbial protein.

PNI is an important indicator for rapidly evaluating the efficiency of dietary nitrogen conversion to MN, with higher PNI values indicating higher efficiency of rumen-degradable nitrogen synthesis into microbial protein. In this experiment, PNI ranged from 0.06 to 0.22, similar to the range (0.07–0.15) reported by Wang et al. [?]. PNI decreased with increasing dietary nitrogen level, consistent with results in Tibetan sheep reported by Zhou et al. [?], although Wang

et al. [?] found that PNI increased with increasing hay intake. The higher PNI under low-nitrogen diets indicates that yaks can effectively utilize rumen-degradable protein to compensate for dietary nitrogen deficiency under nitrogen stress, thereby providing more amino acids to the host.

3.3 Estimation Models for Urinary PD Excretion and Rumen MN Production

The estimation models for small intestine purine absorption and ruminal MN production based on the PD method in ruminant livestock (Table 4 [5-9] and Table 5 [42-45]) show considerable variation in parameters, especially among different species. In this experiment, urinary PD excretion showed a strong linear correlation with estimated ruminal MN production ($R^2 = 0.71$), consistent with estimation models established in various studies shown in Table 5. Reynal et al. [?] found that using ^{15}N as a marker provided higher accuracy for estimating ruminal MN production than the PD method, but Ma et al. [?] also demonstrated high correlation between the two methods ($R^2 = 0.82$), confirming the reliability of the PD method. Estimation of ruminal MN production from urinary PD requires correction of the rumen microbial PN:BN ratio. Perez et al. [?] reported that the PN:BN ratio differs between liquid- and solid-associated microbes, with solid-associated microbes accounting for approximately 70% of the total [?]. Therefore, both solid- and liquid-associated microbes should be extracted in appropriate proportions when determining this ratio. In this experiment, only liquid-associated microbes were considered, so the estimated ruminal MN production may not be entirely accurate. It has been reported that 11% of small intestine-absorbed amino acids originate from protozoa [?], and the PN:BN ratio of bacteria is higher than that of protozoa [?]. During rumen microbial separation, protozoa are easily precipitated with feed particles during centrifugation, and the final separated rumen microbes are mainly bacteria without protozoa [?]. Therefore, using the PN:BN ratio of bacteria to represent that of the entire rumen microbial population may underestimate ruminal MN production.

3.4 Effect of Dietary Nitrogen Level on Urinary Hippuric Acid Excretion

Benzoic acid and glycine are precursor substances for hippuric acid synthesis. Benzoic acid is mainly produced by intestinal bacteria through the degradation of dietary polyphenols in the lower intestine (which are difficult to degrade in the rumen) and is conjugated with glycine in the liver to form hippuric acid [?], thereby preventing benzoic acid toxicity. In this experiment, urinary hippuric acid excretion increased with dietary nitrogen level, consistent with results reported by Liu et al. [?] in Tibetan sheep. Degraded protein in the rumen is the main source of intestinal phenolic compounds [?], and increased dietary nitrogen level increases the main precursors for benzoic acid synthesis, promoting benzoic acid production [?] and consequently increasing hippuric acid

synthesis.

When dietary nitrogen level was 2.85%, ruminal MN production in yaks reached its maximum of 42.60 g/d, while PNI and the efficiency of dietary nitrogen conversion to MN were highest under low nitrogen conditions (1.03%). These results reveal the characteristic of efficient nitrogen utilization from low-nitrogen diets in yaks and explain the nutritional mechanism underlying their adaptation to nutrient-deficient forage on the Qinghai-Tibetan Plateau.

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Note: Figure translations are in progress. See original paper for figures.