

Modulation of feed digestibility, nitrogen metabolism, energy utilisation and serum biochemical indices by dietary *Ligularia virgaurea* supplementation in Tibetan sheep



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ABSTRACT

Ligularia virgaurea is the most widely functional native herbage in the alpine meadow pastures of the Qinghai-Tibet Plateau (QTP) and has multiple pharmacological and biological activities. The effect of *L. virgaurea* as a dietary component on the digestion and metabolism of sheep was evaluated by conducting feeding trials in metabolic cages. Thirty-two Tibetan yearling rams (29 ± 1.56 kg BW) were randomly allotted to four groups included in a completely randomised design with eight animals per treatment. Sheep were fed a basal diet (freshly native pasture) without the addition of *L. virgaurea* (control) or with the addition of *L. virgaurea* (100, 200, or 300 mg/kg BW per day) for 45 days. Addition of *L. virgaurea* to the diet of Tibetan sheep was found to influence the average daily gain (quadratic [Q], $P < 0.001$), feed conversion ratio (Q, $P = 0.002$), CH₄ emissions (linear [L], $P = 0.029$), DM (Q, $P = 0.012$), neutral detergent fibre (Q, $P = 0.017$), acid detergent fibre (ADF) (Q, $P = 0.027$), and ether extract (EE) intake (Q, $P = 0.026$). Apparently, different levels of *L. virgaurea* affected the digestibility coefficients of DM, ADF, and EE (L, $P > 0.05$; Q, $P < 0.05$). The nitrogen (N) intake (Q, $P = 0.001$), retained nitrogen (Q, $P < 0.001$), and N utilisation efficiency (L, $P > 0.05$; Q, $P \leq 0.001$) were also affected by the dietary inclusion of *L. virgaurea*. Effects of *L. virgaurea* feeding were also witnessed on methane energy (CH₄-E) (L, $P = 0.029$), gross energy (GE) (Q, $P = 0.013$), digestible energy (DE) (Q, $P = 0.015$), and metabolisable energy (ME) intake (Q, $P = 0.015$). Energy utilisation efficiency expressed as a proportion of GE intake (DE/GE intake, ME/GE intake, ME/DE intake, FE/GE intake, and CH₄-E/GE intake) manifested quadratic changes ($P < 0.05$) with the increase in the *L. virgaurea* supplementation level. The addition of *L. virgaurea* increased the activity of superoxide dismutase (Q, $P = 0.026$) and glutathione peroxidase activity (Q, $P = 0.039$) in the serum. Overall, the greatest improvement of feed digestibility, N retention, energy utilisation, and antioxidant capacity of Tibetan sheep was yielded by the inclusion of 200 mg/kg BW per day of *L. virgaurea*. Therefore, the addition of an appropriate amount of *L. virgaurea* to the diet of Tibetan sheep is safe and natural, and may enhance the sustainability of small ruminant production systems in QTP areas.

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Implications

As an important component of grassland biodiversity, functional native herbage has important value in animal production. In this study, the application of a functional native herbage—*Ligularia virgaurea* improved the nutrient digestion, energy utilisation efficiency, and nitrogen metabolism efficiency of Tibetan sheep, which has potential production and environmental benefits. Additionally, higher levels of biologically active compounds in

Ligularia virgaurea lead to improved serum antioxidant parameters. Greater use of functional native herbage could be beneficial to reduce reliance on grazing livestock on native pasture and reduce production costs, thereby enhancing the sustainability of local animal husbandry.

Introduction

Traditional grazing land constitutes some 26% of the global total land area and exceeds 80% of the global land area used for forage production, producing over 60% of animal products in both the developed and developing world (Zhang et al., 2015; Mottet

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et al., 2018). However, poisonous plants or weeds are spread freely across 90% of grazed grassland and are traditionally considered undesirable (Molyneux et al., 2014; Kakinuma et al., 2014), and in the traditional grazing model, livestock have a high chance of ingesting these species because they comprise 10–30% of the total biomass of grassland (Cao et al., 2019). As an important component of grassland biodiversity, the presence of these undesirable herbage not only enhances and stabilises grassland productivity and ecological functions but also is a functional native herbage (FNH) of great value to livestock production (Cui et al., 2023a). According to incomplete statistics, the proportion of FNH in rangelands has increased by at least about 10% in the last few decades, while grazing livestock populations have also increased several-fold (Wang et al., 2022). The increase in FNH in grasslands may have enhanced the grassland's capacity to sustain livestock, though the exact dosage and level of intake required to achieve the optimal profit or growth rate in livestock remain unknown. The effects of FNH on animal nutrition metabolism still require further exploration, and further research into how to better capitalise on FNH species is necessary.

With increasing concern and challenges around using antimicrobials in livestock production, there is growing interest in FNH rich in plant secondary metabolites (PSMs) in driving research on plants or by-products of plant origin as dietary additives in animal nutrition for their potential positive effects on livestock productivity and health (Twilley et al., 2020; Yeshe et al., 2022). Many FNHs with low or no known toxicity have been used as feed additives or antibiotic alternatives for livestock to reduce mortality and/or improve growth performance. For instance, *Securigera varia* is toxic to monogastric animals but harmless to ruminants, so it is used as a feed supplement for ruminants in some parts of the world (Al-Snafi, 2016). Supplementation with *Achnatherum inebrians* has been found to improve live weight gain in sheep feeding trials and show no obvious signs of poisoning, indicating its potential as a forage resource (Norton et al., 2009). *Stellera chamaejasme* is known to be toxic, but a study reported its feed value to be equivalent to that of alfalfa, so it can be considered a forage resource once a way is found to detoxify it (Ji et al., 2016). In the foothills and mountain rangelands of western North America, some FNH species have been grazed safely under certain feeding and management conditions (Welch et al., 2015). Moreover, the toxic effects of some FNH species vary with the class or age of livestock. Grazing of *Delphinium spp.* is poisonous to heifers, but is harmless to sheep (Pfister et al., 2013; Green et al., 2019). During the growing season of alpine meadows on the Qinghai-Tibet Plateau (QTP), due to their high nutritive value and the variety of forage options available, grazing livestock generally do not actively feed on these multi-species of FNH unless accidentally ingested or consumed in small amounts for the purpose of treating themselves. At most, the amount ingested does not exceed 5% of the total daily feed (with a maximum of 1% for a single FNH species). During the hay season, most of the FNH present in alpine rangeland contribute to grazing livestock diets, with grazing Tibetan sheep (*Ovis aries*) and yak (*Bos grunniens*) able to ingest multiple FNH species, approximately 5–10% of the total daily feed (though still less than 1% for a single FNH species) (Wang et al., 2022; Cui et al., 2023a). Given the diversity of PSMs in FNH and their potential for complex interactions (mixture effect) upon ingestion, the intake and digestibility of forage may be affected in animals (Acamovic and Brooker, 2005; Welch et al., 2014). Therefore, this has prompted speculation that in grassland regions, there has long been coexistence and adaptation between livestock and FNH: due to the presence of multiple PSMs, when domestic animals consume low doses of FNH, their own regulatory mechanisms may counteract any potential adverse reactions; when in a “safe” context (below the threshold for causing adverse reactions), livestock will consume more of other

grasses to mitigate any discomfort; and when they ingest an amount that causes obvious discomfort, livestock will reduce their feed intake. This strategy of adjusting their feed intake in response to the ingestion of FNH may affect the feed digestibility, physiological response, health, and productivity of livestock (Fig. 1).

FNH deemed undesirable because of the presence of bioactive compounds may in fact impart positive nutrition and health responses in animals. Thus, the present study explored the effects of PSMs and nutrients on the metabolism of livestock by using a PSMs-containing FNH (*Ligularia virgaurea*) typically avoided by ruminants (Tibetan sheep) as an FNH–livestock feed model. *Ligularia* is a genus of FNH in the *Asteraceae* family, widely distributed in grasslands throughout the globe. They contain natural bioactive components—a large family of PSMs (e.g., terpenoids, flavonoids, tannins, saponins) that have anti-inflammatory, antioxidant, analgesic, anti-inflammatory, and other physiologically beneficial properties (Sun et al., 2007; Tori, 2016; Park et al., 2019). Among them, *L. virgaurea* which is widely distributed in the alpine meadows of the QTP, is a traditional, widely used FNH and have been officially recognised as medicinal products used to help prevent or treat gastric ulcers, as well as treat stomachache and nausea (Wu et al., 2004; Okamoto et al., 2010). The objective of the present study was to evaluate the effects of inclusion of *L. virgaurea* in the diet of Tibetan sheep on: (1) productivity, through monitoring average daily gain, feed conversion ratio, feed intake and feed digestibility; (2) environmental sustainability, through estimation of methane emissions, nitrogen metabolism and energy utilisation; and (3) antioxidant and immune properties, through measuring serum biochemical indices.

Material and methods

Study area description

Feed trials were conducted from August to September 2019 at the Maqu Grassland Agricultural Research Station of Lanzhou University (35°58'N, 101°53'E). The research station is located at 3 700 m above sea level, and the average minimum and maximum temperatures are −4 and 8 °C, respectively. The primary rainy season of study area is between June and September, and the average annual rainfall is 620 mm (Ren et al., 2022; Cui et al., 2023b).

Experimental animals, design and feeding

Thirty-two entirely Tibetan yearling rams (29 ± 1.56 kg live weight) were randomly allocated to four dietary treatments, as follows: native pasture (*L. virgaurea*: 0 mg/kg BW (control group); low mass supplementation with *L. virgaurea*, native pasture + *L. virgaurea* (100 mg/kg BW per day); medium mass supplementation, native pasture + *L. virgaurea* (200 mg/kg BW per day); and high mass supplementation, native pasture + *L. virgaurea* (300 mg/kg BW per day).

Feed trials lasted for 45 days, the first 14 of which served as a preliminary period to adapt and last 12 d for data and sample collection (including 6 days of methane measurement and then 6 days of digestibility measurement in metabolic crates). Before the commencement of the experiment, the experimental animals were treated against internal and external parasites using an anthelmintic drug. The experimental animals were kept in individual pens (specially designed to facilitate the collection of urine and faeces), and water was freely accessible. Fresh forage was cut and weighed each day from 30 ha of *L. virgaurea*-free fenced alpine meadow native pasture for feeding Tibetan sheep along with supplementation with *L. virgaurea*. Prior to harvesting the native pasture, the vegetation was assessed by quadrat method and recording the

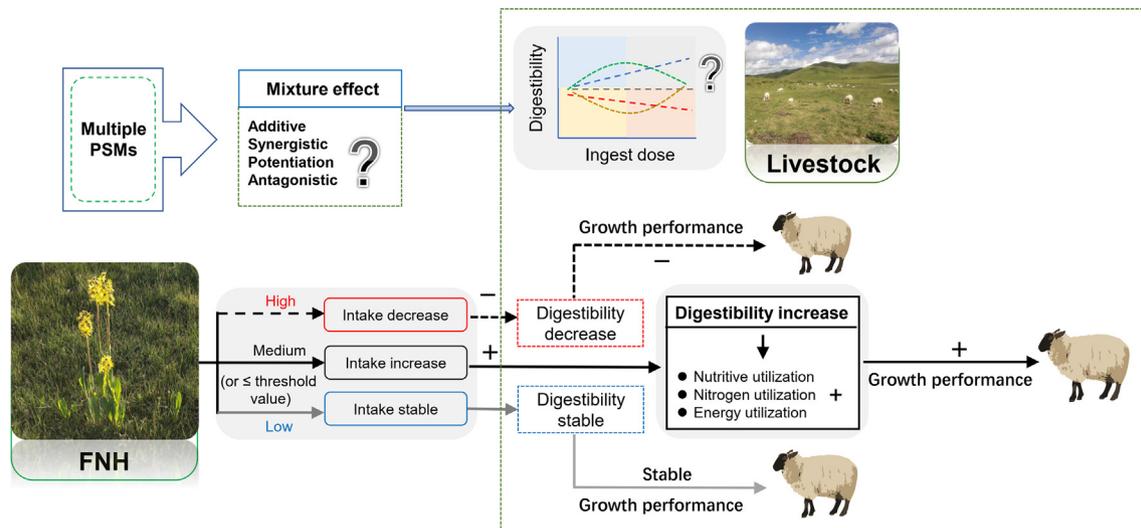


Fig. 1. A hypothesis: the relationship between livestock and functional native herbage. Arrows indicate the feedback loop direction. Plus indicates the positive feedback. Minus indicates the negative feedback. Abbreviations: FNH = functional native herbage; PSM = plant secondary metabolite.

proportion of pasture cover of each dominant family—namely, *Cyperaceae*, *Gramineae*, *Ranunculaceae*, *Compositae*, *Leguminosae*, *Liliaceae*, *Gentianaceae*, and *Scrophulariaceae* (Table 1). Heavily grazed degraded pasture adjacent to the *L. virgaurea*-free fenced alpine meadow native pasture was the source of *L. virgaurea*. The *L. virgaurea* was dried at 50 °C in a forced-air (Kenton GX-130, Guangzhou Kenton Apparatus Co., Ltd.) oven for 48 h, finely powdered in a Willey mill (1 mm) (FZ102, Beijing Zhongxing Weiye Instrument Co., Ltd.), and stored in a tightly closed container and used throughout the experimental period. All basic diets were native pasture, and sheep were fed three times per day at 0800, 1300, and 1800. The fresh native pasture was crushed to ~5 cm in length, and feed amounts were equated to the DM requirement for sheep live weight. The nutritional composition of the basal ration and *L. virgaurea* is shown in Table 1. Due to the hygroscopicity of *L. virgaurea* powder, it was weighed, rolled into pills of equal size and shape, and then dosed with a feeder twice a day (at 0800 and 1300) when offering it to the animals.

Sample collection

The live weights were measured before feeding in the morning on the before the trial (day 0) and final day of the measurement period. During the whole experimental stage, the feed offered to and refused by each sheep was recorded daily to calculate the feed intake (*ad libitum* allowing 10% of refusals).

During the data and sample collection period, the SF₆ tracer gas technique (Johnson and Ward, 1996) was employed to measure enteric CH₄ emissions from sheep over a six-day period. Subsequently, the digestibility measurement period was entered. The bottom of each individual cage was fitted with nylon gauze for

faeces collection, and the subsamples (~10% of total faeces) were taken and composited before the morning feed (approximately 0800 h). Subsamples were mixed with 10 ml of 10% hydrochloric acid, dried in a forced-air (Kenton GX-130, Guangzhou Kenton Apparatus Co., Ltd.) oven at 65 °C for 48 h, ground using a cutting mill (FZ102, Beijing Zhongxing Weiye Instrument Co., Ltd.) equipped with a 1-mm mesh sieve, and stored at -20 °C until chemical analysis. Urine was separated from faeces using a separator attached to the floor of the metabolism cage; 10% of each urine sample was mixed with enough H₂SO₄ to adjust the pH to <3 using a portable pH instrument (PHBJ-260, Shanghai INESA Scientific Instrument Co., Ltd.), before being stored in sterilised plastic bottle in a freezer at -20 °C for analysis of urinary nitrogen (UN) and urinary energy (UE). Ten percent of feed offered and refused as forage was sampled, dried at 65 °C in an oven for 48 h, and then ground through a 1-mm stainless steel screen before being stored in sealed plastic bags for further chemical analysis.

At the end of the feeding trial (day 45), blood samples were collected from the jugular vein of each sheep into serum tubes and then centrifuged at 1 000g for 5 min at room temperature. The separated serum samples were collected and transferred into 2-ml vials and frozen (-20 °C) until analysed.

Analytical methods and calculations

Forage and faeces samples were analysed for DM, organic matter (OM), and ether extract (EE) according to AOAC (AOAC, 2010). Neutral detergent fibre (NDF; assayed using sodium sulphite and with a heat-stable alpha-amylase, and expressed exclusive of residual ash (aNDFom) and acid detergent fibre (ADF; expressed exclusive of residual ash (ADFom)) was determined using a fiber

Table 1
The pasture botanical composition and ingredients and nutritional composition of basal sheep ration and *L. virgaurea* powder (DM basis).

Ingredients	Pasture composition (%)	Components	Pasture	<i>L. virgaurea</i>
<i>Cyperaceae</i>	25.2	DM (%)	36.6	94.6
<i>Gramineae</i>	17.3	Chemical composition (% DM)		
<i>Ranunculaceae</i>	16.8	OM	91.5	92.3
<i>Compositae</i>	15.9	CP	8.5	8.5
<i>Leguminosae</i>	4.5	NDF	56.4	63.3
<i>Liliaceae</i>	4.1	ADF	41.1	50.2
<i>Gentianaceae</i>	2.2	EE	2.1	3.0
<i>Scrophulariaceae</i>	2.1	GE	17.2	17.1
Others	11.9			

Abbreviations: OM = organic matter; EE = ether extract; GE = Gross energy.

analyzer (ANKOM²⁰⁰⁰, ANKOM Technology, Fairport, NY, USA) according to the protocols described by Randby et al. (2010). Forage nitrogen, faecal nitrogen (FN), and UN were measured using the Kjeldahl method (UDK159, VELP), and CP was calculated as Kjeldahl-N \times 6.25 (Dintzis et al., 1988). Meanwhile, the nitrogen balance was calculated by subtracting the total nitrogen excretion (UN + FN) (manure nitrogen, MN) from the total nitrogen (N) intake. Gross energy (GE) was determined by bomb calorimetry (6400, PARR Instrument Co., Moline, IL, USA). CH₄ energy (CH₄-E) was calculated from the CH₄ emissions (L/day) and a conversion coefficient (39.54 kJ/L) (Du et al., 2020). The CH₄ emissions are expressed as the average CH₄ emission (L/day) from 6 days' measurements using the SF₆ (sulphur hexafluoride) tracer technique, carried out as described by Xie et al. (2023). The average daily gain (ADG) was calculated as a difference between final and initial BW divided by the number of feeding days. The feed conversion ratio (FCR) was calculated as the ratio (kg/kg) of the average daily DM intake (DMI) to daily BW change.

An Automatic Biochemical Analyzer (ZY KHB-1280; Shanghai Kehua Bio-Engineering Co., Ltd, Shanghai, China) for total protein (TP), albumin (ALB), globulin (GLB), total cholesterol (TC), triglyceride (TG), creatinine (CREA), Urea nitrogen (UREA), glucose (GLU), aspartate transaminase (AST), and alanine transaminase (ALT) was used. The activity of the serum glutathione peroxidase (GSH-Px), malondialdehyde (MDA), and superoxide dismutase (SOD) was analysed using the corresponding kits (Nanjing Jiancheng Institute of Bioengineering Ltd., Nanjing, China).

Statistical analysis

The effect of the treatment on the response variables was tested by one-way ANOVA (analysis of variance) using IBM SPSS version 25.0 (Statistical Product and Service Solutions (Statistics, IBM Corp., Released 2017. Armonk, NY: IBM Corp). The linear and quadratic contrasts were used to examine the effect of the inclusion of *L. virgaurea* on the response variable. The statistical significance was set at $P < 0.05$, whereas tendencies to differences were accepted if $P < 0.10$.

Results

Growth performance, feed intake and forage digestibility

The effects of different levels of *L. virgaurea* on ADG, FCR, CH₄ emissions, nutrients intake, and digestibility are summarised in Table 2. The ADG, FCR, DM, NDF, ADF, and EE intake followed a quadratic effect ($P < 0.05$) with *L. virgaurea* supplementation levels, while the CH₄ emissions demonstrated linear decreases ($P < 0.05$). Regarding the digestibility, with the increase in the *L. virgaurea* supplementation level, DM, OM, ADF, and EE digestibility also revealed quadratic changes ($P < 0.05$).

Nitrogen metabolism

Table 3 details the nitrogen utilisation of the Tibetan sheep under different treatments. The N intake and retained nitrogen substantiated quadratic changes ($P < 0.05$) with the increase in the *L. virgaurea* supplementation level. Compared with the control, nitrogen excreted via faeces was apparently lower ($P < 0.05$) in the groups receiving *L. virgaurea*, whereas the increase in the *L. virgaurea* supplementation level resulted in a decline in MN ($P < 0.05$). Quadratic changes in the nitrogen utilisation efficiency ($P \leq 0.001$) were observed with the increase in the *L. virgaurea* supplementation level.

Energy utilisation

The energy utilisation of Tibetan sheep under different treatments is illustrated in Table 4. With the increase in the *L. virgaurea* supplementation level, the GE intake, digestible energy (DE), and metabolisable energy (ME) intake documented quadratic changes ($P < 0.05$), whereas the CH₄-E decreased linearly ($P < 0.05$). An increase in the level of *L. virgaurea* addition exerted no effects on faecal energy (FE) output or UE output. Energy utilisation efficiency, expressed as a proportion of GE intake (DE/GE intake, ME/GE intake, ME/DE, FE/GE intake, and CH₄-E/GE intake), also exhibited quadratic changes ($P < 0.05$) with the increase in the *L. virgaurea* supplementation level. Meanwhile, an increase in the level of *L. virgaurea* addition led to no reported effects on UE/GE intake.

Serum biochemical indices

The effects of the different *L. virgaurea* supplementation treatments on the serum biochemical indices of Tibetan sheep are shown in Table 5. Results show that the ALB, TC, TG, UREA, GLU, AST, and MDA were unaffected ($P > 0.05$) by supplementation of *L. virgaurea*. The results reveal that there was a quadratic relationship between the *L. virgaurea* addition level and the TP, GLB, ALT, GSH-Px, and SOD in the serum. Compared with the control, the TP, GLB, and CREA were apparently lower ($P < 0.05$) in the groups receiving *L. virgaurea*, whereas the GSH-Px and SOD were apparently higher ($P < 0.05$), and the highest GSH-Px and SOD values were found in the 200 mg/kg BW group.

Discussion

Up to date a little, if none, data of the effectiveness of FNH on the productive performance of small ruminants on the QTP have been published. This is the first study of the effects of FNH-*L. virgaurea* on growth performance, digestibility, nitrogen metabolism, energy utilisation and serum biochemical indices in growing sheep.

BW gain, feed conversion efficiency and methane emissions

The higher levels of ADG and feed intake achieved by Tibetan sheep consuming a diet supplemented with lower (100 mg/kg) and moderate (200 mg/kg) amounts of *L. virgaurea* compared to lambs consuming native pasture-based diets illustrate the potential benefits of this FNH. Most of the indigenous Tibetan sheep fed on various types of feed attain 20–30 kg live weight with 30–120 g gain/day at yearling age (Liu et al., 2019; Xu et al., 2017). Under traditional grazing conditions, Tibetan sheep generally have slow growth rates (ADG = 30–60 g/day), particularly during the cold season (Sun et al., 2015). As a result, the profitability of the traditional livestock production system, as well as its forage utilisation efficiency, is quite low (Xiao et al., 2020b). This may explain the low ADG and high FCR values of Tibetan sheep in this study. The ADG of Tibetan sheep consumed *L. virgaurea* at 200 mg/kg higher than in the other groups, which is consistent with the findings of a reported change in growth performance, rumen fermentation and immunity in Tibetan sheep receiving *A. membranaceus* (Wang et al., 2021). The FCR is a measure of an animal's efficiency in converting feed mass into body mass (Du et al., 2022). Lower FCR values indicate more efficient feed utilisation, which is essential for animal fattening and growth. The results thus revealed lower FCR values for the group supplemented with 200 mg/kg of *L. virgaurea*, which signifies an improvement in the efficiency of feed conversion in the presence of *L.*

Table 2
The BW gain, feed conversion efficiency, methane emissions and forage quality indicators of the Tibetan sheep fed diets with *L. virgaurea*.

Items	<i>L. virgaurea</i> addition level (mg/kg BW)				SEM	<i>P</i> -value		
	0	100	200	300		ANOVA	Linear	Quadratic
ADG	43.56 ^{ab}	47.73 ^{ac}	52.20 ^c	40.90 ^b	1.012	<0.001	0.700	<0.001
FCR	21.45 ^{bc}	20.35 ^{ab}	19.07 ^a	21.98 ^c	0.305	0.010	0.872	0.002
CH ₄ emissions (L/d)	20.81 ^{ab}	21.34 ^b	18.72 ^a	19.38 ^{ab}	0.361	0.026	0.605	0.874
Intake (g/d)								
DM	929.28 ^{ab}	967.81 ^b	992.42 ^b	896.62 ^a	12.128	0.020	0.508	0.012
OM	707.28	724.29	796.98	726.98	21.286	0.475	0.498	0.482
NDF	463.25 ^a	483.71 ^{ab}	504.87 ^b	453.95 ^a	6.499	0.019	0.910	0.017
ADF	274.4 ^{ab}	286.92 ^{ab}	293.38 ^b	265.46 ^a	3.975	0.049	0.590	0.027
EE	27.26 ^{ab}	29.33 ^{ab}	29.88 ^b	26.69 ^a	0.504	0.059	0.800	0.026
Apparent Digestibility								
DM (g/kg intake)	666.96 ^a	691.11 ^{ab}	705.32 ^b	680.05 ^{ab}	4.222	0.005	0.160	0.002
OM (g/kg intake)	672.50 ^a	693.75 ^{ab}	711.25 ^b	700.00 ^{ab}	4.470	0.011	0.010	0.005
NDF (g/kg intake)	555.94 ^{ab}	562.38 ^{ab}	588.91 ^b	547.39 ^a	5.182	0.021	0.985	0.064
ADF (g/kg intake)	467.97 ^{ab}	473.14 ^b	473.91 ^b	444.89 ^a	4.107	0.031	0.061	0.015
EE (g/kg intake)	519.40 ^a	580.85 ^{bc}	590.69 ^c	537.51 ^{ab}	8.549	0.003	0.410	0.001

Abbreviations: ADG = average daily gain; FCR = feed conversion ratio; OM = organic matter; EE = ether extract.

^{a,b,c} Means in the same row marked with different superscripts differ ($P < 0.05$).

Table 3
The nitrogen utilisation of the Tibetan sheep fed diets with *L. virgaurea*.

Items	<i>L. virgaurea</i> addition level (mg/kg BW)				SEM	<i>P</i> -value		
	0	100	200	300		ANOVA	Linear	Quadratic
N intake and output (g/d)								
N intake	12.58 ^b	12.86 ^b	14.11 ^c	11.55 ^a	0.219	<0.001	0.356	0.001
FN	3.75 ^b	3.33 ^a	3.46 ^a	3.39 ^a	0.059	0.042	0.066	0.055
UN	4.64	4.69	4.43	4.55	0.517	0.317	0.253	0.493
MN	8.39 ^b	8.01 ^{ab}	7.89 ^a	7.94 ^{ab}	0.084	0.126	0.044	0.055
RN	4.19 ^{ab}	4.85 ^b	6.22 ^c	3.62 ^a	0.212	<0.001	0.858	<0.001
Nitrogen Utilization Efficiency								
FN/N intake	0.29 ^b	0.26 ^a	0.25 ^a	0.29 ^b	0.006	<0.001	0.603	<0.001
UN/N intake	0.37 ^b	0.36 ^b	0.32 ^a	0.39 ^c	0.006	<0.001	0.705	0.001
MN/N intake	0.67 ^c	0.62 ^b	0.56 ^a	0.69 ^c	0.010	<0.001	0.957	<0.001
RN/N intake	0.33 ^a	0.38 ^b	0.44 ^c	0.31 ^a	0.010	<0.001	0.957	<0.001

Abbreviations: FN = faecal nitrogen; UN = urinary nitrogen; MN = Manure nitrogen; RN = retained nitrogen.

^{a,b,c} Means in the same row marked with different superscripts differ ($P < 0.05$).

Table 4
The energy utilisation of the Tibetan sheep fed diets with *L. virgaurea*.

Items	<i>L. virgaurea</i> addition level (mg/kg BW)				SEM	<i>P</i> -values		
	0	100	200	300		ANOVA	Linear	Quadratic
Energy intake and output (MJ/d)								
GE intake	15.44 ^{ab}	16.91 ^b	17.16 ^b	14.88 ^a	0.334	0.032	0.645	0.013
FE output	6.70 ^b	6.64 ^b	5.88 ^a	6.35 ^{ab}	0.131	0.098	0.120	0.182
UE output	0.36	0.36	0.38	0.39	0.009	0.703	0.257	0.528
CH ₄ -E	0.82 ^{bc}	0.84 ^c	0.74 ^a	0.77 ^{ab}	0.014	0.026	0.029	0.095
DE	8.74 ^a	10.26 ^{ab}	11.28 ^b	8.54 ^a	0.382	0.025	0.907	0.015
ME intake	7.55 ^a	9.06 ^{ab}	10.15 ^b	8.54 ^a	0.384	0.023	0.864	0.015
Energy utilization efficiency								
DE/GE intake	0.56 ^a	0.60 ^{ab}	0.65 ^b	0.57 ^a	0.013	0.041	0.458	0.036
ME/GE intake	0.48 ^a	0.53 ^{ab}	0.59 ^b	0.49 ^a	0.014	0.035	0.474	0.032
ME/DE	0.86 ^a	0.89 ^{ab}	0.90 ^b	0.86 ^a	0.009	0.053	0.488	0.038
FE/GE intake	0.44 ^b	0.40 ^{ab}	0.35 ^a	0.43 ^b	0.013	0.041	0.458	0.036
UE/GE intake	0.02	0.02	0.02	0.03	0.001	0.239	0.289	0.117
CH ₄ -E/GE intake	0.05 ^b	0.05 ^b	0.04 ^a	0.05 ^b	0.001	0.027	0.244	0.039

Abbreviations: GE = gross energy; FE = faecal energy; UE = urinary energy; CH₄-E = methane energy; DE = digestible energy; ME = metabolisable energy.

^{a,b,c} Means in the same row marked with different superscripts differ ($P < 0.05$).

virgaurea at this level. The high contents of total essential oils (EOs) in *L. virgaurea* could explain, at least in part, this difference in ADG and FCR, as EOs have anabolic properties that enhance feed utilisation efficiency, microbial efficiency, reduce methane production in ruminants (Kholif and Olafadehan, 2021). The increase in feed efficiency shown by the low FCR is one indicator of a decrease in methane production in the rumen. The results of the current study

show that supplementation with *L. virgaurea* at moderate and higher levels may promote reduction in CH₄ emissions; however, the lower CH₄ output at high doses could be partially attributed to the negative impacts of *L. virgaurea* on intake. Moreover, a large variety of natural plants and PSMs (EO [terpenoids], flavonoids, saponins, tannins, etc.) has been explored as ruminant feedstuff for enteric CH₄ mitigation in ruminants (Patra and Saxena, 2010;

Table 5
Effect of *L. virgaurea* supplement on serum biochemical indices in Tibetan sheep.

Items	<i>L. virgaurea</i> addition level (mg/kg BW)				SEM	<i>P</i> -values		
	0	100	200	300		ANOVA	Linear	Quadratic
TP (g/L)	45.43 ^b	34.57 ^{ab}	32.95 ^a	40.98 ^{ab}	1.965	0.019	0.320	0.006
ALB (g/L)	19.60	15.09	17.27	16.70	0.649	0.096	0.270	0.171
GLB (g/L)	25.83	19.48	19.44	24.28	1.048	0.051	0.623	0.020
TC (mmol/L)	1.41	1.26	1.14	1.30	0.070	0.605	0.459	0.417
TG (mmol/L)	0.21	0.21	0.16	0.22	0.011	0.226	0.686	0.427
CREA (umol/L)	42.52 ^a	33.96 ^b	32.84 ^b	34.13 ^b	1.060	0.001	0.004	<0.001
UREA (mmol/L)	3.71	3.60	3.83	3.86	0.113	0.851	0.511	0.771
GLU (mmol/L)	4.53	4.35	4.20	4.32	0.082	0.587	0.291	0.392
AST (U/L)	100.72	75.88	83.17	90.74	6.015	0.524	0.681	0.382
ALT (U/L)	12.43 ^{ab}	9.24 ^a	10.30 ^{ab}	13.49 ^b	0.611	0.047	0.450	0.019
GSH-Px (U/ml)	615.95 ^a	648.40 ^a	763.99 ^b	657.87 ^a	17.618	0.012	0.128	0.039
MDA (nmol/ml)	3.54	3.23	2.54	3.25	0.159	0.145	0.275	0.150
SOD (U/ml)	61.16 ^a	72.08 ^{ab}	83.45 ^b	64.87 ^a	2.965	0.033	0.405	0.026

Abbreviations: TP = total Protein; ALB = albumin; GLB = globulin; TC = total cholesterol; TG = triglyceride; CREA = creatinine; UREA = Urea nitrogen; GLU = Glucose; AST = aspartate transaminase; ALT = alanine transaminase; GSH-Px = glutathione peroxidase; MDA = malondialdehyde; SOD = superoxide dismutase.

^{a,b} Means in the same row marked with different superscripts differ ($P < 0.05$).

Archimède et al., 2016; Albores-Moreno et al., 2017). This reduction in CH₄ production could (partially) be attributed to the high bioactive PSM contents in the *L. virgaurea* used, which may have affected fibre-degrading bacteria and protozoa. This finding substantiates the potential of *L. virgaurea* as a methane-mitigating green additive.

Feed intake and nutrient digestibility

Feed intake can be affected by both external factors and physiological factors, including, animal learning abilities, environmental conditions, and dietary chemical composition (Forbes and Provenza, 2000; Villalba et al., 2006; Xiao et al., 2020a). Moreover, there is a strong correlation between the DM intake and factors affecting the extent of digestion (Keady and Hanrahan, 2015). A plant community with a diverse array of PSMs may allow animals to harvest larger amounts of nutrients; they can have beneficial effects on animal nutrition and health at low doses and in appropriate mixtures (Barrett, 2003). Nonetheless, high doses of these compounds can negatively impact cells and metabolic processes, reducing forage intake (Cheeke, 1988; Athanasiadou and Kyriazakis, 2004). Supplementation of native pasture-based diets with *L. virgaurea* for Tibetan sheep achieved a peak DM intake at the 200 mg/kg supplementation level. Ruminants have a variety of intertwined mechanisms to cope with the consumption of PSM, ranging from physiological (e.g., detoxification pathways) to behavioural (e.g., avoidance, regulation of intake below critical threshold, diet switching, consuming diverse and/or complementary diets) (Estell, 2010). Many studies have found the regulation of feed palatability can be attributed to the secondary metabolites in the plant that consequently alter the forage intake (Athanasiadou and Kyriazakis, 2004; Villalba and Provenza, 2005; Xie et al., 2022). Therefore, it is reasonable to assume that the alteration of forage intake in the current study for the groups supplemented with *L. virgaurea* may have been associated with the level of secondary metabolites, the presence of phytochemicals such as EO, flavonoids, alkaloids, steroids, and saponins could affect the feed intake of sheep. This could be a threshold effect that a high content of *L. virgaurea* supplementation increases the content of dietary PSMs in based diet, which may decrease the diet palatability, so as to decrease the nutrients intake and nutrient digestibility even the growth performance (Makkar, 2003; Wang et al., 2022), which was consistent to our hypothesis.

The apparent digestibility of the DM of herbage is another important parameter to estimate the utilisation of herbage

(Paulsmeier et al., 1995). Amelioration of animal growth is related to the high digestibility of herbage. The increased attention in exploiting natural plants in livestock production, including ruminants, largely depends on the plants' antioxidant properties, modulation of the rumen metabolism, and positive impact on the palatability, feed intake, and digestion (Simitzis, 2017; Liu et al., 2020; Xie et al., 2022). Further investigations into these underexplored FNHs (such as *L. virgaurea*) would help elucidate their added value in augmenting ruminant health and productivity. EOs, including terpenes, aldehydes, alcohols, esters, ketones, and phenols, have been reported in a diverse range of plants, with some being particularly well-known for their high concentrations, such as those of the genus *Ligularia* (Tori, 2016). EOs are applied in animal feed as supplements or additives. In plants, they are involved in several pharmacological activities, but overdosing can lead to detrimental effects. A linear decrease in DM and NDF digestibility with increasing doses of clove oil (from *Eugenia* spp.), eucalyptus oil (from *Eucalyptus globulus*), oregano oil (from *Thymus capitatus*), and peppermint oil (from *Mentha piperita*) mainly by reducing major cellulolytic bacteria, has been reported (Patra and Yu, 2012). Secondary metabolites in *L. virgaurea* that may influence both the rumen microorganism population and its diversity might be partially responsible for the difference in nutrient digestibility in the different treatments in this study.

Nitrogen and energy utilisation

The physiological pathways of metabolism in animals are still largely unknown, meaning there is uncertainty in the potential application of natural plants in animal forage towards achieving desirable effects on the animals' welfare, health, and performance, as well as the quality of the animal product. Just like most terrestrial ecosystems, the QTP is a nitrogen-restricted ecosystem (Yang et al., 2021). However, further experimental research is required to confirm the growth potential of Tibetan sheep and the application potential of FNH, which in itself is crucial in analysing the level of production efficiency in the QTP region and developing sustainable grassland and animal husbandry in the region.

Nitrogen intake is the principal driver of nitrogen excretion from animals. The relevance of the fermentation rate of dietary NDF in determining endogenous nitrogen losses has been highlighted (Marini et al., 2008), with increased nitrogen digestibility resulting in a shift in nitrogen excretion from faeces to urine. The positive effect of slowly fermentable carbohydrates (e.g., NDF) on increased nitrogen output to faeces may be due to the fact that

these carbohydrates can reach the hindgut and provide an energy source for nitrogen-trapping microorganisms, which then pass through faeces rather than urine discharge (Higgs et al., 2012). Inhibition of deamination and methanogens is a result of the addition of some plant extracts to the rumen leading to more efficient utilisation of dietary nitrogen (Calsamiglia et al., 2007). In male sheep, the supplementation of diets with *C. deserticola* at 2 and 4% of the DM intake addition level resulted in improved N intake and deposited N (Liu et al., 2020). Research on plant additives in diets has confirmed that the hydrolysis of plant additives in rumen affects the ability of rumen microbes to capture N, which in turn affects its digestibility and absorption. Improved ammonia-N utilisation in rumen after the addition of *Echium amoenum* extract in the diet of Mehraban lambs has also been reported (Soroor et al., 2013). This may positively influence the environment by reducing urinary N excretion. Feed additives, such as pomegranate peel extract, have been found to redirect excreted N from urine to faeces, and shifting N losses from urine to faeces is expected to minimise N₂O emissions from manure-amended soil, attributed to the lower concentration of volatile MN (Rajabi et al., 2017). In the present study, N intake and retained N manifested quadratic changes with increasing *L. virgaurea* in the diet of Tibetan sheep, and the trend for lower utilisation of dietary N in the 300 mg/kg *L. virgaurea* group is likely associated with lower overall digestibility limiting the synchrony of volatile fatty acids and ammonia necessary for the most efficient microbial protein synthesis. Therefore, the limits of reasonable application should be considered (Rajabi et al., 2017). Notably, there was a significant difference in N egestion in the faeces of Tibetan sheep between the addition groups and control group, and the reduction in FN without increment of MN resulted from the addition of *L. virgaurea* in the diet of the sheep. Consequently, a more environmentally sustainable production was achieved by ameliorating the N efficiency.

Nitrogen excretion may be affected by the key energy parameters, including ME and the ME:GE ratio. The latter is also defined as an indicator of feed quality. Supplementation with *L. virgaurea* declined the loss of energy in faeces and, to some extent, amended the energy utilisation efficiency. Improved DM digestibility could indicate this finding because the less DM excretion there is, the less the FE loss will be (Zhao et al., 2017). A higher DE, ME intake, and ME:GE ratio was obtained in the group of Tibetan sheep fed with 200 mg/kg of *L. virgaurea*. The utilisation efficiency of metabolic energy is attributed to the metabolic capacity. An increase in the amount of rumen fermentable energy may contribute to the high ME and high ME:GE ratio supporting higher rates of microbial protein synthesis, thereby leaving less excess nitrogen and increasing the nitrogen utilisation efficiency (Blaxter and Boyne, 1978; Fanchone et al., 2013). This may suggest that terpenes-rich *L. virgaurea* addition improves the metabolic capacity of N. The addition of terpenes in a sugar beet pulp-based diet in sheep led to a decrease in the acetate concentration and reduced the supply of energy to the sheep, which in turn reduced the ability of lambs to detoxify and eliminate terpenes (Villalba et al., 2006). This signifies an impact of *L. virgaurea* supplementation on the utilisation of metabolic energy for maintenance and the small amount of energy retained in the body. The differences in the overall retention of energy were prominent with and without *L. virgaurea*. It is important to note that CH₄ energy loss as a proportion of GE intake was 4 or 5% for diets with and without *L. virgaurea* supplementation, respectively. The average emissions were much lower than the 6.5% value predicted by the Intergovernmental Panel on Climate Change (IPCC, 2006). These results provided significant information about sheep production in alpine meadows, which is critically important for the IPCC to accurately estimate the global emissions of pastures.

Serum biochemical parameters and antioxidant indices

Serum biochemical indices reflect the magnitude of lipid mobilisation, energy balance, and pathophysiological processes within the body and are a diagnostic marker for health. In the present study, there were no differences observed in ALB, TC, TG, UREA, GLU, AST, and MDA values amongst the treatment means, and no particular pattern was followed. The serum GLB values were significantly influenced ($P < 0.05$) by *L. virgaurea* supplementation, with values ranging between 19.44 and 25.83 for sheep fed with *L. virgaurea*. These values fall within the normal range reported for healthy sheep (Fan et al., 2021). Higher levels of ALT and AST above 45 U/L and 140 U/L could signal hepatotoxicity because these are specific liver enzymes (Oh et al., 2017). This study showed that different dosages of *L. virgaurea* had different effects on transaminase in Tibetan sheep, for which both 100 mg/kg and 200 mg/kg of *L. virgaurea* decreased the serum levels of ALT, while 300 mg/kg increased them. The ALT and AST values in the experimental sheep ranged between 9.2–13.5 and 75.9–100.7 U/L, respectively, and these values are comparable to the value range reported for healthy sheep (Akanmu et al., 2020). The concentrations of these enzymes indicate that no significant metabolic disorder occurred in the sheep. Tibetan sheep fed diets supplemented with *L. virgaurea* had lower serum CREA compared to the untreated group, which led to improved renal function, and was clearly evident in the low blood CREA. The significant decrease in CREA due to treatment with *L. virgaurea* could be associated with the direct modification of nitrogen utilisation in the present study. Antioxidant enzymes such as GSH-Px and SOD present in blood are able to inhibit lipid oxidation of the cell membrane and MDA of the cell membrane oxidation in blood, and their activities in blood are usually used to evaluate the antioxidant ability of animals (Kurata et al., 1993). Meanwhile, the present results showed that sheep fed diets supplemented with *L. virgaurea* succeeded in avoiding such oxidative stress through a significant increase in serum SOD and GPX-Px activities. The positive effect of *L. virgaurea* on blood antioxidant levels may be attributable to the terpenoid content (Graßmann, 2005; Veskoukis et al., 2012). The current result of an increase in SOD and GPX-Px activity indicates that dietary *L. virgaurea* supplementation can improve the antioxidant capacity, although it did not affect the serum activity of MDA in Tibetan sheep. These effects may be attributable to the richness of terpenoid compounds in *L. virgaurea*, thus making them a potential candidate for a natural antioxidant (Bartikova et al., 2014).

Conclusion

The results of this study highlight the beneficial effect of the presence of certain levels of *L. virgaurea* in the diets of Tibetan sheep on forage intake, feed efficiency, nitrogen retention, apparent nutrient digestibility, energy utilisation, and serum biochemical indices. This beneficial impact may reflect the potential of FNH as health modulators towards a collective effort to reduce antibiotic dependence at the pasture scale. These results also suggest the potential application of *L. virgaurea* at 200 mg/kg BW per day in the alpine meadows native pasture diet as a performance enhancer, while simultaneously portraying an improvement in antioxidant properties attributable to higher antioxidant enzyme activities. Therefore, as a promising botanical supplement, *L. virgaurea* has a beneficial effect on the growth, development, and body health of Tibetan sheep, and is also of great importance to forage-saving and increased efficiency in Tibetan sheep production. However, the limited availability of studies hinders a quantitative assessment of the negative and positive effects of *L.*

virgaurea. Further research and long-term studies are essential to validate the impact of individual bioactive chemical compounds contained in *L. virgaurea* on livestock, and to confirm whether its bioactive components are transferred to the animal food products, such as meat and milk. In conclusion, FNH can be used as feeds or antibiotic alternatives for livestock if proper management is conducted. Low or no toxicity FNH species can be used if they have sufficient nutritive value. Feed intake of FNH should be carefully monitored, as too much intake may cause adverse effects. Additionally, more research on the effects of PSMs on livestock and the mechanisms of adaptation between livestock and FNH is needed.

Ethics approval

All animal experimental procedures and handling were approved by the Lanzhou University Research Animal Resource Ethics Committee (File No: 2012-1 and 2012-2).

Data and model availability statement

None of the data were deposited in an official repository. Data are available from the corresponding author upon reasonable request.

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Declaration of interest

The authors declare that there is no conflict of interest.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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