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Phenological Phases of Forage and Rumen Fluid Affect Conjugated Linoleic Acids Composition of Milk of Yak from Qinghai Tibet Plateau

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ABSTRACT

The purpose of this paper was to study the effect of phenological phases on forage nutrient composition, rumen fermentation parameters and content of conjugated linoleic acid (CLA) in milk of grazing yak. In this study, 10 female Gannan yaks with an average body weight of 234.9±10.5 kg were selected for research purpose (natural grazing) in Gannan Pastoral Area of Gansu Province. The forage, rumen fluid and milk samples were collected during green-up time, flowering time and withering time of forage to determine nutrient composition of forage, fermentation parameters of rumen fluid, and CLA in milk fat. The correlation analysis was studied in order to find out the influence of forage and rumen fluid parameters on the content of CLA in milk of grazing yak. The results showed that the phenological phases significantly affected the content of lauric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, total fatty acid, polyunsaturated fatty acids (PUFAs) and total volatile fatty acid (TVFA) (P<0.05), and these were significantly higher in flowering time (P<0.05). The content of fatty acid and total fatty acid in yak milk were significantly higher in flowering time than green-up time and withering time (P<0.05). The phenological phases significantly affected the content of CLA isomer c9t11, t10, c12 and CLA in yak milk (P<0.05), and these were significantly higher in grazing yak milk at flowering time than green-up time and withering times (P<0.05). The rumen pH, acetic acid, propionic acid, acetic/propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, total volatile acid and trans-11-oleic acid in the rumen of yak were also significantly affected by phenological phases (P<0.05). There was a significant positive correlation of rumen pH, forage crude protein, crude fat, nitrogen free extract, tannic acid and total fatty acid with CLA content in grazing yaks (P<0.05). A significant negative correlation (P<0.05) of crude fiber and crude ash with CLA content in milk of grazing yak was recorded, while no significant correlation (P>0.05) of PUFAs and TVFA with CLA content was observed. This study is helpful to improve the grazing management of alpine grassland and the production performance of yaks (milk yield and milk quality).

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Key words
Alpine grassland, Phenological phases, Conjugated linoleic acid, Forage, Rumen fluid, Grazing Yak, *Bos grunniens*, Yak milk, Tannic acid

INTRODUCTION

Yak (*Bos grunniens*) is a unique domestic animal that originated in the Qinghai-Tibet Plateau, China, and is a unique landscape cattle species on the roof of the world, and is also the only bovine subfamily that can breed in the high and cold regions of the Tibetan Plateau (Long *et al*., 2008). Yaks play an important role in the clothing, food, housing, burning (yak dung) and farming of the Tibetan people, therefore yaks are the "omnipotent livestock" in the eye of the Tibetan people. Yak milk is the most important pillar of economic income for the Tibetan people (Jiang *et al*., 2007), and it is also a valuable animal resource in China.

In recent years, it has been found that the type and proportion of fatty acids in food directly affect the occurrence and development of some human diseases (Liu *et al*., 2010). As a new functional fatty acid, CLA has attracted more and more attention (Liu *et al*., 2010). The natural CLA mainly exists in the fat and milk of ruminant such as cattle and sheep. The two isomers of CLA (cis9, trans11 CLA and trans10, cis12 CLA) are present in

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the highest content, and have important physiological functions, such as anti-cancer (colon cancer, stomach cancer, breast cancer, prostate cancer, etc.), disease prevention (atherosclerosis, diabetes, hypertension, etc.), health care, body regulation, fat reduction and weight reduction (Ip *et al*., 2007; Song, 2007).

The studies showed that the content of CLA of cattle milk was affected by many factors, for example, the content of CLA in milk was greatly influenced by the variety of cattle. When feeding the same grass or forage, the contents of CLA in milk were significantly different between different varieties, which were closely related to their different physiological differences. The reason might be that "trans-11" octadecenoic acid and △9 fatty acid desaturase enzyme activity is related (Gao *et al*., 2004; Wang, 2003). The content of CLA in milk fat for grazing ruminants varied significantly with seasons, and the highest values occur in summer and the lowest values occur in winter, which might be related to the composition of nutrients and species of forage (Feng *et al*., 2011). Rumen internal environment, pH value and types of rumen hydrogenated bacteria are also important factors affecting the content of CLA in milk (Han, 2005; Zheng *et al*., 2004). Some ion carrier by inhibiting the growth of some hydrogenated fatty acid bacteria and output of trans oleic acid reduces the content of stearic acid in rumen and increase the content of monounsaturated fatty acids, and thus increasing the content of CLA. In the aspect of animal feeding, the amount of intake, the type of fat content in feed, the level and type of cellulose in diets, the content of copper in diet, the proportion of green forage and the way of feeding have certain effects on CLA in milk (Xue *et al*., 2004; Li *et al*., 2006). In terms of molecular genetics and cell theory, studies mainly focused on the effects of enzymes related to fatty acid synthesis on CLA production in ruminants, such as: fatty acid synthetase (FAT), acetylCoA carboxylase (ACC), stearoyl coenzyme A dehydrogenase (SCD), etc., in addition, some research on gene polymorphism and expression of related genes has been conducted (Li *et al*., 2006; Zhe *et al*., 2013). In recent years, tannic acid has been considered as an antinutritional factor, it has been found that adding a certain amount of tannic acid to diets has a certain positive effect on CLA in milk or meat of ruminants.

In this study, we determined the composition of CLA in milk fat of grazing yaks during different phenological phases, aiming to find out the influence of forge phenological phase on the composition of CLA in milk fat of grazing yaks. We correspondingly measured the conventional nutrient, tannic acid and fatty acids in forage grass, while rumen pH and rumen fermentation parameters such as volatile fatty acid (VFA), trans-11-oleic acid (TVA) in rumen were also measured in different forge phenological phase. Correlation analysis of the CLA in milk fat with other parameters was also conducted to explore their effects on CLA. The purpose of this paper is to study the effect of herbage phenology phases on the composition of fatty acids and the content of CLA in grazing yak milk.

MATERIALS AND METHODS

Research area

This study was conducted in alpine meadow of the Qinghai-Tibet Plateau to achieve research objectives. The grassland belongs to the eastern part of the Qinghai-Tibet Plateau (33°06′ ~ 35°32′ north latitudes, 100°44′ ~ 104°45′ east longitude) located in Maqu County, Gansu Province. The climate of area is cold and humid type, with a long cold season and a short warm season. The annual average temperature is -0.5°C to 3.5°C, the extreme maximum temperature is 28°C, and the extreme minimum temperature is -23°C. The average annual rainfall is 545 mm, concentrated in July, August and September. The surface runoff depth is 200-350 mm, and the annual evaporation is 1 222 mm. Alpine grassland is rich in plant species with 12-22 species per m^{-2} in this vegetation meadow. The main dominant species are perennial Cyperaceae, Polygonaceae, Rosaceae and Fabaceae species, such as *Kobresia humilis*, *Poa pratensis* L., *Elymus nutans* Griseb. and *Polygonum viviparum* L.

Experimental design

In this experiment, 10 naturally grazed calving Gannan yaks were selected from herdsmen's pastures around the northeast edge of the Qinghai-Tibet Plateau, which were healthy in physique, similar in age (6-8 years old), similar in parity (from the end of April to the beginning of May). The site of 12 ha area alpine meadow were chosen to grazing by 10 naturally grazed calving Gannan yaks with their calves from May to December every year. The grazed alpine meadow was fenced for the whole experiment period, and grazed from 1 May to 31 December with a stocking rate of 0.83 yak/ha (Yao *et al*., 2019). Slope, aspect, elevation and soil type of all sites are similar to 14°, Sunny slope, 3840m and alpine meadow soil respectively. Grazing time of alpine meadow from May to December were divided three phenological phases, including green-up time (May), flowering time (September) and withering time (December) (Li *et al*., 2019). At this grazing site, there were thirty 100 m × 100 m stationary monitoring blocks (approximately 50 m away from each other) were established with the same conditions for collecting forage samples in three phenological phases. We also tracked and collected the yak milk, rumen fluid and grazed forage during three growth stages. Every effort was made to minimize animal pain, suffering and distress and to reduce the number of animals used. Gannan yaks management followed traditional practice, with grazing yaks were kept in the grazing sites day and night and were allowed to drink freely. We measured the CLA content in milk and conventional nutrients, PUFAs, and tannic acid (TD) in grazed forage. While, volatile fatty acids (VFA), trans-11-oleic acid (TVA), and pH were measured in rumen fluid. We analyzed the influence of phenological period on the content of CLA in Gannan yak milk, and explored the factors influencing CLA content of milk in Gannan yak. Animal welfare and experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), and the protocol was approved by the committee of the Institute of College of Agriculture and Animal Husbandry of Qinghai University. Every effort was made to minimize animal pain, suffering and distress and to reduce the number of animals used.

Forage sample collection

The site of grazing alpine meadow with uniform vegetation grazed by yak was selected in the three phenological phases and successive samplings were collected once in the middle of each month of three phenological phases (in green-up time (May), flowering time (September) and withering time (December) respectively). 30 samples of 1 m × 1 m size were selected from the site with uniform vegetation. The number of plant species in each quadrat were investigated, recorded and all plant species in each quadrat were excised to the ground. The samples were separately packed into labeled envelope bags, and taken to the laboratory. The samples were dried to constant weight in an oven at 65°C, crushed with a three-dimensional crusher, and then passed through a 1 mm mesh screen for forage nutrient and tannin content determination.

Milk and rumen fluid samples collection

The cows and calves were isolated overnight (to prevent breast-feeding). At 08:00 the next morning, the milk and rumen fluid of yaks were collected before feeding. The milk and samples were collected in 50 ml centrifuge tube. To collect rumen fluid, the yaks were put down and bound, sample was extracted and stored in 50 ml collecting tube. 50 mL milk and rumen fluid were transferred immediately to the laboratory and stored at -80 ℃.

Determination of forage nutrients and tannic acid (TD) content

Dry matter (DM) was determined by drying method (Čop *et al*., 2009), crude protein (CP) was determined by automatic Kjeldahl nitrogen analyzer (Kheltec 8400, Foss Company, the United States), crude fat (EE) was determined by traditional Soxhlet extraction method (Čop *et al*., 2009), crude fiber (CF) was analyzed by fully automatic fiber analyzer (ANKOM 2000 Fiber Analyzer, Foss Company, the United States) and crude ash was calcined. Nitrogen-free leachate content was calculated as 100% - (moisture + crude protein + crude fat + crude fiber + crude ash) (Gao *et al*., 2004), total calcium was measured by complex titration method, total phosphorus was determined by national standard spectrophotometry and TD content was determined by ultraviolet spectrophotometer (UV-2700, Shimadzu Corporation, Japan) (Čop *et al*., 2009; He *et al*., 2019).

Determination of fatty acids in forage, milk and rumen fluid

The content of milk protein, milk fat, TS, SNF, lactose, casein, FFA, citric acid, density and acidity in yak milk was determined by automatic milk composition analyzer (UV-2550, Shimadzu Corporation, Japan). The CLA in yak milk was extracted, methylated and analyzed by gas chromatography, prior to its determination by normalization method and acetylchloride methanol methylation method. The fatty acid extraction and methylation from forage and rumen fluid samples were done by using milk fat analysis methods (Baiyila, 2002). The rumen pH was determined by pH meter (M250782, Shanghai Chunye Instrument Technology Corporation, China) and VFA were analyzed by gas chromatography (GC-2010 plus, Shimadzu Corporation, Japan) (Zhou *et al*., 2011).

Data processing

Microsoft office 2010 was used to organize and plot data, while SPSS 19.0 statistical software (IBM Corp., Armonk, New York, USA) was used for statistical analysis. The three phenological phases of forage with different nutritional quality indicators, fatty acid composition, milk quality indicators of grazed yaks and the content of CLA were analyzed by single factor analysis of variance. Significant differences of the effects of each individual predictor were accepted at $P<0.05$.

RESULTS

Forage nutrients and TD content

The phenological phases showed a significant effect on the content of DM, CP, EE, CF, ash, NFE, Ca, P, Ca/P and TD ($P<0.05$). The content of Ca/P of forage in green-up time was significantly higher than flowering time and withering time ($P<0.05$). In comparison to green-up time, the content of ash in withering time decreased by 30.2% while, the rate of Ca/P decreased by 16.5% (Table I). The content of CP, EE, Ca, P and TD were significantly higher in flowering time than green-up time and withering time (P<0.05). The content of Ca and TD were significantly higher in green-up time and flowering time than withering time (P<0.05). Compared to withering time, the content of CP, EE, Ca, P and TD in forage of flowering time increased by 66.5%, 66.3%, 42.9%, 44.4% and 60.8%, respectively (Table I). The content of DM, CF and NFE in withering time were significantly higher than green-up time and flowering time (P<0.05). In comparison to green-up time, the content of DM and CF in forage of withering time increased by 3.0% and 23.5%, respectively.

Table I. Effect of phenological phases on forage nutritional value and tannic acid (%, dry matter basis).

Index	Green-up time	Flowering time	Withering time	SEM	P value
DM	90.59±0.28[c]	91.87±0.63[b]	93.38±0.58[a]	0.33	<0.01
CP	6.97±1.22[b]	10.76±1.51[a]	3.60±0.49[c]	2.59	0.01
EE	1.51±0.69[b]	2.70±0.37[a]	0.91±0.35[b]	3.14	0.02
CF	22.34±3.93[b]	22.96±1.20[b]	29.21±0.88[a]	1.55	<0.01
Ash	9.06±1.48[a]	8.37±1.35[a]	6.32±1.56[b]	0.94	0.01
NFE	50.26±3.81[b]	47.07±2.32[b]	52.34±2.52[a]	1.12	0.03
Ca	0.39±0.04[a]	0.42±0.05[a]	0.24±0.06[b]	0.26	<0.01
P	0.14±0.01[b]	0.18±0.01[a]	0.10±0.01[c]	0.00	0.01
Ca/P	2.79±0.04[a]	2.33±0.05[b]	2.40±0.06[b]	0.11	0.03
TD	1.57±0.10[a]	1.66±1.15[a]	0.65±0.13[b]	1.31	<0.01

DM, dry matter; CP, crude protein; EE, crude fat; CF, crude fiber; NFE, nitrogen free extract; TD, tannic acid. In the same row, values with the same letters mean no significant difference (P>0.05), while with different letters mean significant difference (P<0.05). The same pattern is used throughput the paper.

Forage PUFAs content

The phenological phases displayed significant effect on the lauric acid, tetradecanoic acid, cetylic acid, octadecanoic acid, linoleic acid, linolenic acid, TFA, PUFAs and PUFAs/TFA (P<0.05) (Table II). The contents of lauric acid, tetradecanoic acid, cetylic acid, linoleic acid, linolenic acid, TFA, PUFAs and PUFAs/TFA in forage of flowering time were significantly higher than green-up time and withering time (P<0.05) (Table II). The contents of octadecanoic acid in forage of withering time was significantly higher than green-up time and flowering time (Table II). Compared to withering time, the content of lauric acid, tetradecanoic acid, cetylic acid, linoleic acid, linolenic acid, TFA and PUFAs increased by 100%, 100%, 14.1%, 34.7, 49.3%, 30.2% and 34.5%, respectively in flowering time (Table II). In comparison to green-up time, the content of octadecanoic acid in forage of withering time increased by 39.0%, and the rate of PUFAs/TFA of forage increased by 9.8% in flowering time (Table II).

Milk components of grazing yaks

The phenological phases of forage had a significant effect on the milk protein, milk fat, TS, SNF, lactose, casein, FFA, citric acid, density and acidity content (P<0.05), and showed no significant effect on the milk urea and FPD (P>0.05) (Table III). Compared to green-up time, the content of milk protein, SNF, lactose, casein, FFA and density decreased by 28.4%, 17.3%, 24.1%, 19.0%, 56.4% and 0.7%, respectively in withering time (Table III). In comparison to flowering time, the content of milk fat, TS and acidity of grazed yak decreased by 36.1%, 20.4% and 23.6%, respectively and the citric acid content increased by 43.6% in withering time (Table III).

CLA content in milk of grazing yaks

The phenological phases of forage displayed a significant effect on the content of c9t11 CLA, t10c12 CLA and TCLA (P<0.05) (Table IV). The contents of c9t11 CLA, t10c12 CLA and TCLA in milk of flowering time were significantly higher than green-up time and withering time (P<0.05) (Table IV). Compared to flowering time, the contents of c9t11 CLA, t10c12 CLA and TCLA in milk decreased by 67.7%, 64.7% and 66.7%, respectively and these contents were lowest in the withering time (Table IV).

Content of volatile fatty acids in rumen fluid of grazing yaks

The phenological phases of forage significantly affected the pH value, acetic acid, propionic acid, acetic acid/propionic acid ratio, isobutyric acid, butyric acid, isovaleric acid, valeric acid, TVFA and TVA of rumen in yaks (P<0.05) (Table V). The rumen pH, the contents of isobutyric acid and valeric acid of green-up time were significantly higher than grassy and withering time (P<0.05); the contents of TVFA and TVA of flowering time were significantly higher than withering time (P<0.05); the contents of acetic acid, propionic acid, butyric acid, valeric acid, TVFA and TVA of flowering time were significantly higher than green-up time and withering time (P<0.05); the contents of butyric acid, valeric acid and TVA of green-up time were significantly higher than withering time (P<0.05); the ratio of acetic acid/propionic acid of withering time was significantly higher than green-up time and flowering time (P<0.05), while green-up time had significantly higher ratio than flowering time (P<0.05) (Table V).

Table II. Effect of forage phenological phases on composition and content of polyunsaturated fatty acid (mg/g) in forage.

Index	Green-up time	Flowering time	Withering time	SEM	P-value
Lauric acid (C12:0) (mg/g)	0.40±0.02[b]	1.03±0.13[a]	0.00±0.00[c]	0.24	<0.01
Tetradecanoic acid (C14:0) (mg/g)	0.94±0.05[b]	1.32±0.08[a]	0.00±0.00[c]	1.59	<0.01
Cetylic acid (C16:0) (mg/g)	18.74±0.70[a]	18.79±0.81[a]	16.15±1.22[b]	0.68	0.03
Octadecanoic acid (C18:0) (mg/g)	3.86±0.21[c]	4.88±0.25[b]	6.33±0.33[a]	0.22	<0.01
Linoleic acid (C18:2) (mg/g)	4.39±0.72[b]	6.52±0.62[a]	4.26±0.87[c]	0.14	<0.01
Linolenic acid (C18:3) (mg/g)	15.84±1.09[b]	21.14±1.81[a]	10.72±1.58[c]	3.54	<0.01
Total fatty acids (TFA) (mg/g)	44.17±1.06[b]	53.68±2.51[a]	37.45±3.12[c]	6.18	<0.01
Polyunsaturated fatty acid (PUFAs) (mg/g)	24.09±1.26[b]	32.54±1.89[a]	21.31±2.29[c]	4.26	0.02
PUFAs/TFA %	0.55±0.02[c]	0.61±0.01[a]	0.57±0.02[b]	0.67	0.02

In the same row, values with the same letters mean no significant difference (*P*>0.05), while with different letters mean significant difference (*P*<0.05).

Table III. Effect of forage phenological phases on composition of grazing yak milk.

Index	Green-up time	Flowering time	Withering time	SEM	P-value
Protein (% of DM)	5.88±0.96[a]	5.60±0.62[a]	4.21±0.48[b]	1.28	<0.01
Fat (% of DM)	6.68±2.05[a]	6.95±1.89[a]	4.44±1.52[b]	0.37	0.02
TS (% of DM)	17.71±2.64[a]	18.00±1.64[a]	14.32±1.39[b]	0.22	<0.01
SNF (% of DM)	11.63±1.33[a]	11.53±1.13[a]	9.62±0.70[b]	1.59	0.01
Lactose (% of DM)	5.53±0.82[a]	5.53±0.74[a]	4.20±0.41[b]	2.33	<0.01
Casein (% of DM)	4.85±0.80[a]	4.63±0.48[a]	3.93±0.54[b]	0.41	0.01
FFA (% of DM)	3.12±1.95[a]	1.50±1.10[b]	1.36±0.76[b]	3.47	<0.01
Citric acid (% of DM)	0.23±0.03[b]	0.22±0.04[b]	0.39±0.58[a]	0.64	0.03
Urea (% of DM)	0.03±0.01[a]	0.01±0.01[a]	0.03±0.02[a]	0.85	0.54
Density (g/cm^3)	1036.50±1.48[a]	1032.80±1.66[b]	1029.9±1.73[c]	0.05	0.04
Acidity (°T)	12.75±2.72[a]	13.59±1.30[a]	10.38±1.29[b]	1.32	0.01
FPD (°C)	0.71±0.06[a]	0.76±0.08[a]	0.71±0.06[a]	1.63	0.67

TS, total solid; SNF, solid no fat; FFA, free fatty acid; FPD, freezing point depression. In the same row, values with the same letters mean no significant difference (*P*>0.05), while with different letters mean significant difference (*P*<0.05).

Table IV. Effect of forage phenological phases on conjugated linoleic acid content of grazing yak milk (mg/ml).

Index	Green-up time	Flowering time	Withering time	SEM	P-value
c9t11 CLA (mg/ml)	0.23±0.06[b]	0.34±0.07[a]	0.11±0.02[c]	2.35	<0.01
t10c12 CLA (mg/ml)	0.11±0.03[b]	0.17±0.04[a]	0.06±0.02[c]	1.29	<0.01
TCLA (mg/ml)	0.34±0.09[b]	0.51±0.11[a]	0.17±0.04[c]	3.22	0.02

c9t11-CLA, c9t11 conjugated linoleic acid; t10c12 CLA, t10c12 conjugated linoleic acid; TCLA, total conjugated linoleic acid. In the same row, values with the same letters mean no significant difference (*P*>0.05), while with different letters mean significant difference (*P*<0.05).

Correlation analysis of the conjugated linoleic acid in milk with other parameters

The CP, NFE, TD and TVFA of forage and pH value of rumen fluid showed a significant positive correlation with CLA content of milk in grazing yaks (*P*<0.05) (Fig. 1). The CF and ASH of forage displayed a significant negative correlation with CLA content of milk in grazing yaks (*P*<0.05) (Fig. 1). The PUFAs content of forage showed positive significant correlation with CLA content of milk in grazing yaks (*P*>0.05) (Fig. 1).

Table V. Effects of forage phenological phases on rumen fermentation parameters and of grazing yak.

Index	Green-up time	Flowering time	Withering time	SEM	P-value
pH value	6.26±0.21[a]	6.12±0.16[a]	5.82±0.20[b]	1.28	<0.01
Acetate (mmol/L)	40.22±8.07[b]	58.99±12.72[a]	47.55±9.98[b]	0.37	0.02
Propionate (mmol/L)	8.17±1.65[b]	13.95±3.6[a]	9.20±1.84[b]	0.22	<0.01
Acetate/ Propionate	4.94±0.41[b]	4.29±0.35[c]	5.16±0.38[a]	1.59	0.01
Isobutyric (mmol/L)	0.51±0.10[a]	0.47±0.05[b]	0.44±0.10[c]	2.33	<0.01
Butyrate (mmol/L)	6.34±1.38[b]	9.05±2.13[a]	4.75±0.56[c]	0.41	0.01
Isovaleric (mmol/L)	0.76±0.24[a]	0.45±0.13[b]	0.48±0.10[b]	3.47	<0.01
Valeric (mmol/L)	0.45±0.09[a]	0.54±0.15[a]	0.26±0.03[b]	0.64	0.03
TVFA (mmol/L)	56.46±11.17[b]	83.46±18.48[a]	62.69±12.25[b]	0.85	0.04
TVA (mg/g)	2.88±0.32[a]	3.26±0.69[b]	1.06±0.20[c]	0.05	0.04

TVFA, total volatile fatty acid; TVA, Trans-11-oleic acid. In the same row, values with the same letters mean no significant difference (P>0.05), while with different letters mean significant difference (P<0.05).

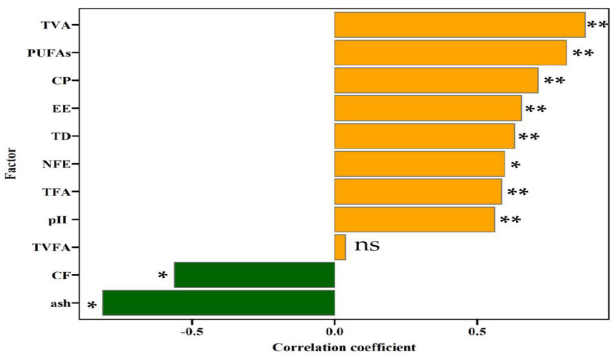

Fig. 1. Correlation analysis of CLA in forage, milk, and rumen of grazing yak.
CP, crude protein; EE, crude fat; CF, crude fiber; NFE, nitrogen free extract; TD, tannic acid; TFA, total fatty acid; PUFAs, polyunsaturated fatty acid; TVFA, total volatile fatty acid; TVA, trans-11-oleic acid; * P>0.05; ** P< 0.01; ns, not significant.

DISCUSSION

Influence of phenological phases on nutrition and PUFAs in forage grass

The contents of CP, CF, ASH, EE, Ca, P and Ca/P ratio are the primary factors that influence the nutritional value of forage grass (Frame, 2001). Based on the results obtained in this study, the phenological phases exerts a significant effect on the nutrient content of forage grass. The nutritional value of forage grass in the flowering time was higher than green-up time and withering time. The contents of NFE and CF in forage grass during the withering time were found to be significantly higher than green-up time and the flowering time. Consistent with the results in this study, Zhao *et al*. (2012) found that the nutrient content of forage during the warm season in the Qinghai-Tibet Plateau was much higher than the cold season. The CP content and crude ash in the warm season were significantly higher than the cold season, while the CF content in the cold season was remarkably higher than that in the warm season. Through evaluating the nutritional value of different forages on the Qinghai-Tibet Plateau, Zhao *et al*. (2013) found a significant negative correlation between the CP content and the CF content. By studying the nutritional value of forage grass in Tibet, Zhao *et al*. (2012) discovered that the content of nutrients in forage grass varies with the seasons i.e., the forage grass in summer featured the best quality of nutrients, which could met the nutritional needs of local grazing livestock, while the quality of forage was the lowest in winter and spring with a shortage of CP content. Based on the research results, the content of CP in forage was mainly determined by the climatic conditions. When the air humidity coefficient decreased and the climate dryness coefficient increased, the content of CP in forage showed significantly high content (Chen, 1985). Gannan Tibetan Autonomous Prefecture had abundant rainfall, sufficient light and high temperature in summer, but low temperature and insufficient sunshine in winter, which was closely correlated with the low content of CP in forage grass. If the content of acid detergent fiber was too high (≥30%), it would affect the digestion of CP, and the high content of CF in winter seriously affected the nutritional value and palatability of forage grass (Wang *et al*., 2019, 2020). Study reported that the contents of Ca, P and Ca/P ratio were of great significance to the growth, development and metabolism of livestock (Wang *et al*., 2020). It was concluded in this study that changes in conventional nutrient contents such as Ca, P and CP followed a specific pattern in different growth periods of forage grass i.e., the quality of forage grass in the flowering time surpassed the green-up time and withering times, while forage grass in the green-up time enjoyed a higher quality than that in the withering time (Li *et al*., 2019). There are few studies on the seasonal variations of the content of tannin which is an anti-nutritional factor. Based on the results obtained in this study, the phenological phases exerts a significant effect on the content of tannin of forage grass, the content of tannin in forage grass during the green-up time and flowering times were significantly higher than that in the withering time, and there was no significant difference between the green-up time and flowering times in terms of the content of tannin. Cao (2016) studied the content of TD in *Bashania mangosteen* and the results showed that the content of TD in mangosteen varied with the altitude and season. The content showed an upward trend from the start of spring in February to the mid-summer in July and August, with the highest content recorded in July and August and the lowest in February. By measuring the content of TD in *Casuarina equisetifolia* in Chishan Forest Farm of Dongshan County, Fujian Province, Zhang *et al*. (2009) found that the content of TD in young and mature twigs in summer were remarkably higher than winter.

The composition and content of fatty acids in forage grass are affected by season, region, weather and precipitation. PUFAs are one of the most important precursors for ruminants to synthesize CLA (Baiyila, 2002). A high content of PUFAs in forage grass promotes the synthesis of CLA and increases the deposition of CLA in ruminants, Linoleic acid is one of the precursors for ruminants to synthesize CLA (Baiyila, 2002; Feng *et al*., 2011). The study by Feng *et al*. (2011) on the contents of fatty acids in 28 kinds of forage grasses reported that there were some differences in the contents of fatty acids among different kinds of forage grasses, in which the content of linolenic acid (C18:3) was the highest, cetylic acid (C16:0) and linoleic acid (C18:2) came to the second place, and the contents of other fatty acids were relatively low. This implied that the species and growth of forage varied from one region to another due to climatic factors. As a result, the contents of various fatty acids in forage grass were different (Feng *et al*., 2011; Fu *et al*., 2013). Fu *et al*. (2013) explored the composition of fatty acids in natural mixed forage grass in Yanchi County, Ningxia Province and the results showed that PUFAs content in mixed forage grass accounted for 36%-57% of the TFA content in May, and the PUFAs content increased 46%-61% of the TFA content in October. Based on the experimental results, PUFAs accounted for about 55% of the TFA in the green-up time (May) and increased 61.0% of the TFA in the flowering time (September), which was in line with the findings obtained in this experimental study i.e., the PUFAs content was characterized by an upward trend from the green-up time to the flowering time. Zhu *et al*. (2013) measured the content of fatty acids in Tianzhu Zangzu Autonomous County and the results showed that the PUFAs content in summer and autumn was generally higher than that in winter and spring. These results are consistent with the experimental results gained in this study. The possible reason is that both Tianzhu Zangzu Autonomous County and Gannan Tibetan Autonomous Prefecture are located in the Qinghai-Tibet Plateau and have similar altitude and climatic environment, so the species and growth cycle of forage grass are similar as well.

Influence of phenological phases on milk composition and CLA content of grazing yak

As a functional fatty acid, CLA has been a hot research object in recent years for its special efficacy (Liu *et al*., 2009). The CLA in ruminants is hydrogenated by linoleic acid (C18:2) in the rumen or transformed from the intermediate product (TVA) generated by the hydrogenation of PUFAs in feed (Yin *et al*., 2012). The results showed that CLA displayed obvious seasonal differences (Tuo *et al*., 2013). According to some experimental studies, the CLA content in the milk of grazing yaks during the flowering time was significantly higher than the withering time, which is consistent with the results obtained in this study. The CLA content in the flowering time significantly surpassed the green-up time and withering times, and the CLA content in the green-up time was obviously higher than that in the withering time. It was probably because of the good nutritional quality of forage in summer and autumn as well as the abundant nutrients in the forage grass during this growth stage provided the grazing yak with more nutrients. The results showed that the CLA content in fatty acids fluctuated the most with the change in seasons (Lock *et al*., 2004), with the highest content found in the flowering time and the lowest content found in the withering time during winter. It was found that △9 desaturase was more active in the flowering time in summer, which showed that the forage grass in the flowering time could increase the activity of △9 desaturase in the mammary gland of lactating cows, supporting the results gained in this experiment. The study carried out by Xue *et al*. (2004) showed that the fundamental reason for the difference in CLA content in the cow's milk was the amount and quality of fresh green forage eaten by the cow. Besides, a large number of studies (Xue *et al*., 2004; Fu *et al*., 2005; Zhang *et al*., 2011; Sheng and Wu, 2012) revealed that daily ration played an essential role in the CLA content of cow's milk, which further supported the conclusion that the total contents of c9t11-CLA and t10, c12-CLA in the milk of grazing yaks in the flowering time were significantly higher than those in the green-up time and withering times. As reported by Gao *et al*. (2004), drinking milk is very important for us to maintain the health benefits of our body, a person weighing 70 kg needs about 3 g of CLA per day. In our research, our body needs for CLA every day shows a significant change with phenological stages, 883 ml, 588 ml and 1 760 ml yak milk respectively have been intaken in the green-up time, the flowering time and the withering time, respectively.

Influence of phenological phases on VFA and CLA contents in the rumen of grazing yak

Previous studies (Sheng and Wu, 2012) revealed that the rumen pH is affected by many factors, e.g. dietary composition and dietary intake. Based on the results obtained in this study, the rumen pH during the green-up time and flowering times was significantly higher than that in the withering time. This was probably related to the changes of nutrients in forage and the dietary intake of the grazing yak, resulting in changes in the total VFA content and pH value of the rumen. There are few studies on the seasonal dynamic changes of VFA in the rumen fluid of grazing yaks. In this experiment, we measured the contents of VFA in the rumen fluid of lactating yaks in 3 forage phenological phases, i.e. green-up time, flowering time and withering time. The results showed that the highest acetate content in the rumen fluid of grazing yaks was found in the flowering time, and it was significantly higher than the green-up time and withering times. The contents of propionate, butyrate, valeric, TVFA and TVA also shared the same variation trend, with the highest content found to be in the flowering time, which were significantly higher than the green-up time and withering times. This is probably because of the different nutrient contents in forage and the dietary intake of grazing yaks caused by distinctive climatic environments in different seasons, which might leads to the changes inside the rumen environment, thereby resulting in significant variation in the contents of the above-mentioned substances. According to previous studies, the proportion of acetate in fermented green forage reached above 70% (Fu *et al*., 2005), which is consistent with the results in this study. Zhang *et al*. (2011) used meteorological chromatography to measure the VFA content in the rumen fluid of yaks, pien niu (offspring of a bull and a female yak) and yellow cattle on the Qinghai-Tibet Plateau during the warm and cold seasons, finding that the total VFA content in the rumen fluid was significantly different between warm and cold seasons and the ratio of acetate to propionate ranged from 3.9 to 5.0, both of which belonged to acetate fermentation. This is consistent with the conclusion gained in this study i.e., the total VFA content in the rumen fluid of grazing yaks in Gannan Tibetan Autonomous Prefecture during the warm season was significantly higher than cold season, and rumen fermentation in the green-up time and flowering times belonged to acetate fermentation. TVA is an important intermediate product of biohydrogenation in ruminant milk fat. Recent studies indicated a significant correlation between the content of TVA in ruminant milk fat and the content of CLA in ruminants and TVA produced CLA in ruminants. This study showed that as the phenological phases changed, the content of linoleic acid (C18:2) and TVA in forage grass during the green-up time and flowering times were significantly higher than withering time. Studies showed that increasing the content of linoleic acid (C18:2) in feed would lead to an increase in the content of TVA in the rumen fluid of dairy cows and beef cattle (Zhang *et al*., 2013; Guo *et al*., 2019). Yang (2017) also found that since soybean oil is rich in linoleic acid (C18:2), adding 2.0% soybean oil to feed could significantly increase the content of fatty acids, TVA and CLA in the rumen fluid, which is in line with the results obtained in this experiment.

Correlation between fatty acids in rumen fluid and forage with content of CLA in milk fat

Tannin has always been regarded as an anti-nutritional factor (Kelly *et al*., 1998). According to recent studies, adding appropriate amount of TD to the diet of ruminants could increase the CLA content in ruminants (Yang, 2017). Based on the results gained in this study, there was a certain correlation between the content of TD in forage grass and the content of CLA in yak milk. Kelly *et al*. (1998) found that TD played a strong role in inhibiting hydrogenated bacteria of fatty acids in the rumen. Since sheep feeds on coronal rock astragalus forage rich in tannin, the content of linolenic acid (C18:3) in intramuscular fat was 4 times higher than that in the control group, with the content of C22 -pentaenoic acid in long-chain fatty acid increased as well. By adding 45% flaxseed powder (rich in tannin) to the diet of lamb, Valentina *et al*. (2009) found that the ratio of CLA to trans-11-octadecene in the fatty acids of lamb muscle increased remarkably, and the expression of △9 desaturated fatty acid protease in the lamb muscle also showed an increasing trend.

Based on the study by Wahle-Klaus *et al*. (2004), CLA was produced by TVA in ruminants with the help of △9 dehydrogenase, so TVA was the precursor in the rumen to synthesize CLA. According to this study, as the phenological phase varied, the TVA content in the rumen fluid of yaks was linearly correlated with the CLA content in yak milk, and the two shared a similar variation trend i.e., the contents of CLA and TVA in the flowering time were significantly higher than those in the green-up time and withering times and the contents in the green-up time were significantly higher than those in the withering time. This agrees with the experimental results in this study. Pu (2006) also found a linear correlation between the TVA content in the rumen of cows and the CLA content in their milk.

According to Valentina *et al.* (2009), insufficient dietary fiber leads to a decrease of pH value in the rumen, which affected the formation of TVA (the precursor in the rumen to synthesize CLA), thereby influencing the content of CLA in milk of grazing yaks. In this study, from the green-up time to the withering time, the level of CF showed a gradual upward curve, which was not correlated with the change of CLA content in milk of grazing yaks. This was possibly because of the poor quality of forage grass caused by the weakened palatability as a result of the high content of CF. Based on the results obtained in this study, the pH of the rumen fluid during the withering time was lower than 6.0, which was significantly lower than that in the green-up time and flowering times. No significant difference was found between the flowering time and the green-up time. The pH value of the rumen fluid was correlated with the CLA content in milk of grazing yaks to some extent Pu (2006). Lin *et al.* (2010) found that linoleic acid isomerase had the activity of transforming and synthesizing CLA when the pH value varied from 5.5 to 8.5, and the decrease of pH value in the rumen inhibited the activity of cellulose decomposing bacteria, thereby weakening the hydrogenation of PUFAs, reducing the TVA content in the rumen and decreasing the CLA content in milk of grazing yaks. According to the study by Martin and Jenkins (Martin and Jenkins, 2002), when the pH value in the rumen was lower than 6.0, the CLA content in the milk declined significantly; as the pH value rised, the CLA content increased as well.

CONCLUSIONS

Phenological phases had significant effects on the composition and content of fatty acids in forage, rumen fluid and milk fat of grazing yak. The contents of polyunsaturated fatty acids (PUFAs) in forage, rumen fluid and milk fat of grazing yak were the highest in the flowering time and the lowest in the withering time. The contents of PUFAs in forage and rumen fluid were significantly correlated with the milk fat. It is suggested that grassland grazing is best carried out in the flowering time of phenological stage, which can helpful to improve the grazing management of alpine grassland and obtain a higher production performance of yaks (milk yield and milk quality).

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Statement of conflicts of interest

The authors have declared no conflict of interest.

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