Association between dietary components and muscle fatty acid deposition in longissimus dorsi: Results from a metabarcoding diet analysis of grazing Tan sheep

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Abstract

Understanding the natural diets of grazing herbivores can help fulfill their nutritional requirements and develop management strategies. Emerging metabarcoding techniques can provide more accurate estimates for dietary composition of grazing animals. Thirty-nine Tan sheep with weights of 25.10 ± 1.88 kg were randomized into three groups: the grazing group, the time-limited grazing group, and the stall-fed group. Effects of grazing on meat fatty acid composition in lambs were compared to concentrate-based systems. Simultaneously, we investigated sheep diets using DNA metabarcoding of feces to assess the prevalence of medicinal herbage plants consumed by grazing sheep. Metabarcoding data determined that Lespedeza sp., Artemisia sp., Chenopodium sp., Corispermus sp., and Phellodendron amurense were predominant with different proportions ($P < 0.05$). Our results demonstrated that grazing systems could transform the muscle fatty acid composition and promote n-3 PUFAs, including C18:3n3 (ALA), C20:5n3 (EPA), and C22:6n3 (DHA) deposition. To establish the association of PUFAs with the herbage taxa, we conducted multivariate and correlation analyses. Some highlighted herbage species (e.g., Bassia scoparia, Euphorbia humifusa, and Arnebia euchroma) were significantly correlated with omega-3 PUFAs. The dominant group Lespedeza sp. showed a positive correlation with C18:2n6. Overall, these results demonstrated the utility of metabarcoding diet analysis and how diversification in dietary composition was associated with muscle fatty acid deposition. This research examined the correlation between herbage taxa and omega-3 fatty acids, and the results provide an initial view of the effects of herbage on PUFAs of lambs. The study provides experimental evidence for future feeding research.

Association between dietary components and muscle fatty acid depositionin*longissimus dorsi*: Results from a metabarcoding diet analysis of grazing Tan sheep

Running title: Metabarcoding in sheep diet and PUFAs

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Abstract

Understanding the natural diets of grazing herbivores can help fulfill their nutritional requirements and develop management strategies. Emerging metabarcoding techniques can provide more accurate estimates for dietary composition of grazing animals. Thirty-nine Tan sheep with weights of 25.10 ± 1.88 kg were randomized into three groups: the grazing group, the time-limited grazing group, and the stall-fed group. Effects of grazing on meat fatty acid composition in lambs were compared to concentrate-based systems. Simultaneously, we investigated sheep diets using DNA metabarcoding of feces to assess the prevalence of medicinal herbage plants consumed by grazing sheep. Metabarcoding data determined that *Lespedeza sp.* , *Artemisia sp.* ,*Chenopodium sp.* , *Corispermus sp.* , and *Phellodendron amurense* were predominant with different proportions ($P < 0.05$). Our results demonstrated that grazing systems could transform the muscle fatty acid composition and promote n-3 PUFAs, including C18:3n3 (ALA), C20:5n3 (EPA), and C22:6n3 (DHA) deposition. To establish the association of PUFAs with the herbage taxa, we conducted multivariate and correlation analyses. Some highlighted herbage species (e.g., *Bassia scoparia* , *Euphorbia humifusa* , and *Arnebia euchroma*) were significantly correlated with omega-3 PUFAs. The dominant group*Lespedeza sp.* showed a positive correlation with C18:2n6. Overall, these results demonstrated the utility of metabarcoding diet analysis and how diversification in dietary composition was associated with muscle fatty acid deposition. This research examined the correlation between herbage taxa and omega-3 fatty acids, and the results provide an initial view of the effects of herbage on PUFAs of lambs. The study provides experimental evidence for future feeding research.

Keywords: Herbivore, sheep, diet, feces, muscle PUFAs, meat flavor

1 | INTRODUCTION

With the global demand for healthy foods increasing, sheep meat production and consumption are gaining greater importance worldwide. Sheep production in the form of meat (lamb or mutton) makes a significant contribution to the economic activity of many countries. Consumers require meat that is safe, of consistent eating quality, healthy, and convenient (Nuernberg, Fischer, Nuernberg, Ender, & Dannenberger, 2008). The consumers' preference for sheep meat is mainly due to its nutritious features and healthy fatty acid composition (de Andrade et al., 2017).

Indeed, sheep meat is one of the major sources of long-chain n-3 polyunsaturated fatty acids (PUFAs) in human diets along with fish, fish oils, and eggs (Beng et al., 2016). The n-3 PUFAs, mainly alpha-linolenic acid (ALA, C18:3n3), eicosapentaenoic acid (EPA, C20:5n-3), and docosahexaenoic acid (DHA, C22:6n-3), are beneficial to human health (Aglago et al., 2020). These beneficial n-3 PUFAs have significant roles in maternal and childhood brain development, optimal cardiovascular function, and retinal functions (Pewan et al., 2020). The composition of meat fatty acids in grazing sheep strongly differs from that of sheep raised

under barn feeding conditions. Pasture feeding can promote absorption and deposition of C18:3n3 from grass in the intramuscular tissue and can decrease the ratio of n-6/n-3 fatty acids compared to concentrate feeding (Nuernberg, Fischer, Nuernberg, Ender, & Dannenberger, 2008; Zhang, Jin, Badgery, & Tana, 2017). While some studies have investigated fatty acid variation in different feeding systems, little is known about the association between dietary diversity and variability in fatty acid composition.

Quantitative estimates of dietary composition of herbivores in the context of complex plant distribution in the grassland are essential to determine herbivore-herbage preference and to investigate the relationships between performance of herbivores and their diets. Measuring the diet choices of grazing animals presents several challenges complicating the evaluation of feed efficiency in grassland ecosystems (Vargas Jurado et al., 2019). Traditionally, diet analyses of grazing herbivores have been conducted through n-alkanes and long-chain alcohols of plants detected from animal feces (Zhang et al., 2019). However, their use in identification of diet items of grazing herbivores is not always accurate (Lin et al., 2012; Narvaez, Brosh, & Pittroff, 2012). Application of a molecular approach to quantify the diet composition can lead to greater understanding of how changes in the diet affect production (Pompanon et al., 2012; Kowalczyk et al., 2019; Waraniak, Marsh, & Scribner, 2019). Metabarcoding has been suggested as a useful tool for determining animal diets due to its greater accuracy and resolution (Bhattacharyya, Dawson, Hipperson, & Ishtiaq, 2019). Metabarcoding is conducted through utilizing conserved gene regions to amplify sequences in samples that are unique in different taxa (King, Read, Traugott, & Symondson, 2008). Still, the use of metabarcoding to study diets of grazing herbivores has been relatively limited.

Here, we focus on the applicability of dietary metabarcoding as a sampling tool for examining the diet composition of Tan sheep grazing in a desert grassland. Additionally, we evaluated the effects of grazing type compared to concentrate feeding on muscle fatty acid composition, particularly n-3 PUFAs, in the *longissimus dorsi* (LD). This study also explored herbage-PUFAs correlation profiles in lambs through systematic correlation analyses. The results provide an initial view of herbage-PUFAs association in fatty acid accumulation that will promote further understanding of the flavor-relevant deposition process.

2 | MATERIALS AND METHODS

2.1 | Study area and animal management

The study was conducted over an 83-day period, including a 10-day adaptation period and a 73-day treatment period, from August 1st to October 22nd, 2019. The experiment took place in natural desert steppe located behind the Tan Sheep Breeding Farm (106°58'E, 37deg26'N; alt. 1400 m) in the eastern region of Ning Xia of northwestern China. The mean annual temperature of the area is 8.3degC, and average annual precipitation is 282.3 mm, occurring mostly between June and September. The predominant native plant species were *Lespedeza sp.* followed by *Caranana sp.* during the experimental period. Other forage is also present in the meadow, including *Salsola sp.* and*Artemisia sp.* , but with irregular distributions. A total of 40 hectares of pasture were fenced off into eight equal paddocks used as rotational grazing plots.

All animal experiments received approval from the China Agricultural University Laboratory Animal Care Advisory committee. All experimental procedures and animal care protocols were accomplished in accordance with the guidelines provided by the Institutional Animal Care Advisory Committee for China Agricultural University. Thirty-nine healthy male lambs of Tan sheep with an average initial body weight of 25.10 +- 1.88 kg were randomly divided into three treatment groups (n = 13): a stall-feeding group (control group), a time-limited grazing group (LG), and a grazing group (G). The lambs began to access pasture at 7:00 and were removed at 11:00 and 19:00 for 4h (LG) and 12h (G) treatments, and then were separately housed in individual pens at the end of grazing. All of the lambs from the stall-feeding group were individually penned in steel cages. The lambs were fed their respective diets twice per day at 8:00 and 17:00 h, and the lambs for the LG treatment only received their supplementary feed at 17:00 h. The basal diet was formulated with concentrates and hay (10% alfalfa hay, 15% corn stover, 75% TMR) to meet or exceed the nutrient requirements from the National Research Council (NRC 2007); the ingredients are listed in Table 1. During the 10-day adaptation period, feed was adjusted daily based on the previous day's intake, allowing refusals

of 15%. All sheep were provided with adequate water and salt blocks throughout the experimental period.

2.2 | Sample preparation

For the different grazing time analysis, fecal samples were collected twice from 12 of the 26 individuals from 3 September (LG1 and G1) and 7 October in 2019 (LG2 and G2). At each of the two time points, six lambs from each treatment were placed in cages before grazing to collect feces. The fresh feces samples from each lamb for each period were collected and were immediately stored at -20degC. Three days of fecal samples were pooled for each lamb during each sampling period and mixed fully as a compound sample for subsequent molecular analysis.

At the end of the experimental period, all lambs were transported to an abattoir and were slaughtered after 24-h fasting by exsanguination from the jugular vein. Within 45 minutes of slaughter, the LD muscle was sampled at the level of the 10–13th rib from the side of the carcass and frozen at -20degC for fatty acid (FA) analysis.

2.3 | Analysis of the fatty acid composition

FA composition of freeze-dried LD muscle samples was quantified using Agilent 6890 gas chromatography mass spectrometry (GC/MS) (Agilent Technologies Inc., Wilmington, DE). The FAs were methylated with acetyl chloride/methanol (1:10, v/v), and hendecanoic acid methyl ester (C11:0) was used as an internal standard (1mg ml^{-1}). The DB-23 column (60 m x 0.25 mm, film thickness 0.25 μm) temperature was held at 130°C for 1 min and then was increased to 260°C at a rate of 4°C min^{-1}. Subsequently, the injector and detector (FID) temperatures were set at 270°C, and the helium flow ratio was 2 ml min^{-1}. An automatic split injector was used with a ratio 30:1. By comparing the retention times of the individual FA peaks with the known standards (37 component FAME mixture), the fatty acids could be identified.

2.4 | Molecular analyses protocol

The metabarcoding diet analyses based on sheep feces (n = 24) were used to estimate dietary herbage items in different grazing treatments. All aspects of the laboratory molecular protocol consisted of DNA extraction, amplification of the ITS2 fragment, and amplicon sequencing on a MiSeq run.

Total DNA was extracted from lamb feces samples using an E.Z.N.A. Stool DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer's instructions. Concrete operations were conducted as described by Guo et al. (2018). The DNA extract was checked on 2% agarose gels, and DNA concentration and purity were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA).

The ITS2 region of the nuclear rDNA (350 bp) was amplified with primer pairs rD5-ITS2 (5'-barcode-TCCTCCGCTTATTGATATGC-3') and rb1-ITS2F (5'- CGATACTTGGTGTGAATTGCAG-3') (Bradley et al., 2007) by an ABI GeneAmp$^{(r)}$ 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of the ITS2 gene was performed as follows: initial denaturation at 94 for 5 min followed by 45 cycles of denaturing at 94 for 30 s, annealing at 59 for 60 s and extension at 72 for 60 s, and single extension at 72 for 10 min ending at 4. The PCR mixtures contained 4 μL of 5 × FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of forward primer (5 μM), 0.8 μL of reverse primer (5 μM), 0.4 μL of*TransStart* FastPfu DNA Polymerase, 10 ng of template DNA, and finally ddH_2O up to 20 μL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using a Quantus Fluorometer (Promega, USA).

Purified ITS2 amplicons were pooled in equimolar volumes and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) according to standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). These sequence data have been submitted to the GenBank databases under accession number PRJNA660588.

2.5 | Bioinformatics processing of sequencing data

The raw ITS2 gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 (Chen, Zhou, Chen, & Jia, 2018) and merged by FLASH version 1.2.7 (Magoc, & Salzberg, 2011) using the following criteria: (i) The 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded; reads containing ambiguous characters were also discarded. (ii) Only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap regions was 0.2. Reads that could not be assembled were discarded. (iii) Samples were distinguished according to the barcode and primers. Exact barcode matching was specified, with a two nucleotides mismatch in primers matching being permitted.

Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE version 7.1 (Edgar, 2013) , and chimeric sequences were identified and removed. The taxonomic identity of OTUs was annotated using a BLAST search of the reference set of OTU sequences against public databases (GenBank and EMBL) using a similarity threshold of >95% for species-level identification. Furthermore, final taxonomic classification was based on the closest blast match as well as other considerations involving the geographical locations of the species and the diversity of closely related species (Deagle, Chiaradia, McInnes, & Jarman, 2010). Sequences were advised to be allocated to a higher taxonomic level (e.g., genus or family) when the same score was assigned to two or more taxa for this sequence.

2.6 | Diet composition computation

The analytic protocols of OTUs refinement were performed by way of referring to the description in Shutt et al. (2020). First, only OTUs belonging to the plant kingdom were considered as possible food items. Uncorrelated nonfood OTUs (e.g., fungi, bacteria, or Metazoa) were removed. All OTUs identified as environmental contamination (e.g., algae) were removed. Then, all OTU reads with fewer than 0.01% of the total were removed as possible false positives. The above steps reduced the number of sequence reads from 2,463,964 to 1,426,517 containing 92 OTUs. Finally, all remaining OTUs belonging to the same best-match taxon (at the genus or species level) were merged (remaining n = 56).

Plant taxa and their respective numbers of sequences in each sample were summarized. Samples from the stall-feeding treatment were excluded from analyses. Multiple metrics were used to interpret the diets of Tan sheep. Those considered here are Occurrence data and Read abundance data (Deagle et al., 2019).

Occurrence Data, including percent frequency of occurrence (%FOO), percent of occurrence (POO), and weighted percent of occurrence (wPOO), were calculated as

$$\%FOO_i = \frac{1}{S}\sum_{k=1}^{S} I_{i,k} \times 100\%,$$

$$\mathrm{POO}_i = \frac{\sum_{k=1}^{S} I_{i,k}}{\sum_{i=1}^{T}\sum_{k=1}^{S} I_{i,k}},$$

$$\mathrm{wPOO}_i = \frac{1}{S}\sum_{K=1}^{S} \frac{I_{i,k}}{\sum_{i=1}^{T} I_{i,k}},$$

where T is the number of plant items, S is the number of samples, and I is an indicator function such that $I_{i,k} = 1$if plant item i is present in sample k , and 0 otherwise. FOO analysis was based on a 0.01% threshold (Alberdi, Aizpurua, Gilbert, & Bohmann, 2018).

Read abundance data using the sequence counts were used to calculate relative read abundance (RRA_i) for plant item i as follows:

$$\mathrm{RRA}_i = \frac{1}{S}\sum_{k=1}^{S} \frac{n_{i,k}}{\sum_{i=1}^{T} n_{i,k}} \times 100\%,$$

where $n_{i,k}$ is the number of sequences of plant item i in sample k .

2.7 | Statistical analysis

The effects of three feeding patterns on muscle fatty acid composition were compared by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at the 95% confidence level. A rarefaction curve and Venn diagram based on OTU matrices were generated using Mothur 1.30.2. Principal co-ordinates analysis (PCoA) and distance heatmap plots were employed to visualize variation in botanical community across samples. To examine trends in RRA of items in the sheep diet, the data set was reduced to RRAs of the twenty most dominant taxa, which together comprised over 97% of all plant taxa sequences. Then, we conducted two-way ANOVA using GLM with Duncan's multiple range test to identify significant differences in RRA of predominant plant items between grazing patterns and grazing periods. These statistical analyses were performed with SPSS Statistics version 17.0 (IBM, Armonk, NY). The fatty acid and RRA profiles were reported as mean values ± SEM.

Additionally, multivariate statistical analyses were conducted to interpret the relationships between variables and treatments using CANOCO for Windows version 4.56 (Leps, & Smilauer, 2003). The main gradients of variation in grazing treatments were described by principal component analysis (PCA). Redundancy analysis (RDA) was performed to determine the responses of herbage taxa and fatty acids to grazing treatment. The treatment combinations of herbage taxa (based on RRA metrics) and the treatment combinations of muscle PUFAs at the end of growing season were used as response variables for PCA and RDA. Monte Carlo tests were conducted in the RDA with restricted random permutations of samples. The correlation matrix was calculated in both PCA and RDA. Pearson correlation coefficients were also employed to analyze the relationship between diet taxa and muscle PUFAs. $P < 0.05$ was considered statistically significant. Figures were generated with OriginPro version 9.5.1 software (OriginLab Corporation, USA).

3 | RESULTS

3.1 | Fatty acid composition of *longissimus dorsi*

The relative contents of muscle FAs obtained from the 39 lambs and the corresponding statistical analysis are shown in Table 2. The highest percentage FA was oleic acid (C18:1n9), ranging from 33.49% (grazing group) to 37.93% (stall feeding group). All FAs, with the exception of SFA (C10:0, C12:0, C14:0, C17:0), MFA (C14:1, C16:1, C24:1, C22:1n9), PUFA (C20:2, C18:2n6), significantly varied across the three feeding modes (Table 2). The grazing treatments for 4 h or 12 h increased ($P < 0.01$ or $P < 0.05$) the proportions of SFAs (i.e., C13:0, C18:0, C21:0, C22:0, C23:0, and C24:0), but resulted in a lower ($P = 0.002$) C16:0 proportion compared to the control group. In addition, lower ($P = 0.012$) proportions of C18:1n9 were found in the grazing and time-limited grazing treatments. The effect of the feeding regime on the FA content was calculated, and the data indicated that the grazing treatments clearly improved almost all PUFAs. For example, the grazing treatment had the highest proportions of C18:3n3 (α-linolenic acid, ALA) and C20:5n3 (timnodonic acid, EPA) compared to the other two treatments ($P < 0.001$). Compared to the other feeding modes, the grazing treatment had a higher ($P < 0.001$) proportion of C22:6n3 (docosahexaenoic acid, DHA) than the other treatments, which were not significantly different from each other. The grazing treatment had the highest ($P = 0.041$; $P = 0.004$) proportions of C20:3n6 and C20:4n6 and tended ($P = 0.102$) to have increased C18:2n6. Compared with the control, the sum of n-3 polyunsaturated fatty acids (PUFAs) was markedly increased, and the ratio of n-6/n-3 was decreased from 17.11% to 7.69% after grazing for 4 h or 12h ($P < 0.001$).

3.2 | Raw read quality of DNA sequencing

Metabarcoding dietary analysis successfully amplified sequences of plant items from 12 Tan sheep lambs. Of the total fecal compound samples (n = 24) collected, all samples amplified the target ITS2 region. A total of 2,463,964 raw pair-end reads were obtained from the MiSeq run after demultiplexing and quality filtering. Irrelevant phyla, e.g., Ascomycota and Chlorophyta, were also generated, but our diet analysis focused only on phytogroups. Of these, 1,429,952 sequences were retained composing of 324 OTUs. Of those, 92 OTUs

with a high percentage of reads (99.76%) were conserved (threshold: $> 0.01\%$ of the total). Rarefaction curves for each individual sample reached saturation in all cases (Figure 1a). The grazing and time-limited grazing samples had 68 OTUs in common (Figure 1b).

3.3 | Diet characterization

Plant sequences from the samples were gathered into 56 taxa based on the lowest taxonomic classification (genus or species level) that could be determined confidently from GenBank and EMBL. The most abundant sequences in the pool of potential food items were those related to*Lespedeza sp.* (30.78%). Next were those related to*Artemisia sp.* (16.27%), *Chenopodium sp.* (14.44%), and*Corispermum sp.* (11.83%). In total, 26–44 diet items derived from different taxa (mean = 35) were present in the diets of lambs.

PCoA via the Binary-Jaccard algorithm of diet communities revealed that diets segregated mainly by preference for a handful of plant species (Figure 1c). For all the diet item plant communities, a slightly higher dependency on grazing type than grazing period was the most important driving factor contributing to the variation in diet composition, explaining 18.25% of the variation. The second most important distinction in diet composition was the different compositional herbage species changing with the growing season. ANOVA results indicated that there was significant interaction between the grazing modes and sampling period ($R^2 = 0.50$, $P = 0.001$). The predominant diet items in all samples are summarized and shown in the heatmap (Figure 1d). In general, the dominant genera in different grazing types and within the same modes in different periods had numerous differences.

The top 20 taxa accounting for 97.96% of the total sequence reads were used to investigate the diet composition of Tan sheep. Overall,*Lespedeza sp* ., *Artemisia sp* ., *Chenopodium sp* ., and*Corispermum sp* . were found to be the most predominant herbage groups, being present in 100% of samples (FOO) and comprising 73.30% of the total of diet DNA sequences (RRA) (30.81%, 16.29%, 14.40%, and 11.80%, respectively). *Phellodendron amurense* , *Medicago sativa* , *Salsola sp* ., *Bassia scoparia* , *Euphorbia humifusa* , *Eragrostis sp* ., *Ixeris tamagawaensis* ,*Setaria italica* , and *Cenchrus flaccidus* were also present in 100% of the samples, comprising 3.94%, 3.32%, 3.14%, 2.51%, 2.05%, 1.47%, 1.23%, 0.78%, and 0.61% of RRA, respectively. Other plant taxa included *Sibbaldianthe bifurca* at 95.83% (1.05% RRA) and *Arnebia euchroma* at 91.67% (1.59% RRA). *Oxytropis halleri* , *Chloris pilosa* , *Convolvulus sp* .,*Astragalus peterae* , and *Caragana korshinskii* were present at 83.33%, 33.33%, 91.67%, 75.00%, and 91.67% FOO, respectively (< 1% RRA; Table 3, Figure 2).

The multiple RRA measurements were averaged for each type-stage combination. As shown in Figure 3, the stacked bar charts indicate that the prevalent genera in the grazing group were basically the same as those in the time-limited grazing group, dominated by species such as*Lespedeza sp.* , *Artemisia sp.* , *Chenopodium sp.* , *Corispermum sp.* , and *Phellodendron amurense* , whereas the proportions quite different ($P < 0.05$, Figure 3 and Table 3).

3.4 | Relationships between diet composition and fatty acid

To interpret the influences on fatty acid deposition, we conducted an association analysis to map important taxa in the desert steppe. Principal component analysis was performed to characterize the correlation structure among response variables in grazing type treatments (Figure 4 and Table 4). The eigenvalues of the first and second axes were 0.399 and 0.227, respectively. Axis 1 was dominated by *Corispermum sp.* , *Salsola sp.* , and*Bassia scoparia* with positive values (score values: $> |0.7|$) and *Artemisia sp* . and *Chenopodium sp* . with the negative values (score values: $> |0.7|$). Axis 2 was primarily explained by three n-3 PUFAs indicators with positive values (score values: $> |0.5|$) and *Euphorbia humifusa* , *Arnebia euchroma* , *Oxytropis halleri* , and*Ixeris tamagavaensis* with positive values (score values: $> |0.6|$).

RDA was conducted to further assess the association in the data set relative to all treatments (Table 4 and Figure 5). Two axes explained 59.7% of the total variance. The grazing treatment was markedly different from the time-limited grazing treatment, with more plentiful herbage species and greater n-3 fatty acids (RDA axis1,$R^2 = 0.220$, $F = 6.20$, $P = 0.002$).*Arnebia euchroma* , *Euphorbia humifusa* , *Salsola sp.*

,*Bassia scoparia* , *Oxytropis halleri* , and *Ixeris tamagaxaensis* positively influenced n-3 PUFAs compostion via C18:3n3, C20:5n3, and C22:6n3.

Finally, we constructed 171 herbage-PUFAs pairs consisting of 12 herbage species and 6 n-3 PUFAs. Figure 6 shows the interaction patterns among herbage-PUFAs pairs. Table S1 lists the correlation coefficients of these pairs. Of the 171 pairs, 47 (27.5%) were correlated at $P< 0.05$ or $P < 0.01$, corresponding to 10 unique herbage taxa. The correlation coefficients ranged from -0.410 to 0.959, among which *Salsola sp.* , *Bassia scoparia* ,*Euphorbia humifusa* , and *Arnebia euchroma* exerted significant influence on the n-3 PUFAs. We found that C18:2n6 was positively correlated with *Lespedeza sp* . ($P < 0.05$).

4 | DISCUSSION

The present work is a pioneering study of how the deposition of PUFAs in lamb muscle can be explained by diet items in desert grassland habitats. The present study introduces a novel and in-depth interpretation of the field diet consumed by grazing sheep that benefits from the power and robustness of fecal metabarcoding. Our findings qualitatively confirm the hypothesis that certain specific herbage taxa, not always the visible dominant species, are one of the determinants of the flavor formation in meat products of sheep.

4.1 | Diet characterization

The determination of diet composition of large grazing herbivores has been a difficult problem due to methodological limitations and habitat complexity. In comparison with traditional diet analysis of herbivore fecal samples, metabarcoding approaches provide a fast and high-resolution identification to species level (Vesterinen et al., 2016). Both frequency of occurrence (FOO) and relative read abundance (RAA) were applied to obtain semi-quantitative diet data. Overall, our survey of sheep feces revealed 56 plant taxa (at genus level or species level) (Figure 2). Both approaches have shown accurate estimates when the number of food taxa in samples is small (Mcinnes et al., 2017). However, there are taxa biases in recovery of sequences. FOO tends to overestimate the importance of food groups with small amounts. RRA can reflect more accurate relative proportions of the population-level diet.

Metabarcoding dietary analysis allows quantification of variation in animal diets due to changes in usability and taxa composition (Table 3 and Figure 3). The herbage taxa, such as *Lespedeza sp* .,*Caragana korshinskii* , *Oxytropis sp.* , *Salsola sp.* ,*Bassia sp.* , *Setaria italica* , and *Cenchrus sp.* , in the diets of Tan sheep as characterized by metabarcoding data were broadly consistent with our knowledge base for the diets of Tan sheep in this area. The high diversity of food taxa observed in sheep diets can, to a large extent, be attributed to the capability of metabarcoding to quickly detect plant items derived from food residues that are often hard to identify by other conventional techniques. Sequencing data based on RRA metrics demonstrated that lambs presented variant diets as a response to grazing type or grazing period, with approximatively all food taxa assigned to the species level.

The selection of dietary components is affected by many factors such as habitat characteristics and availability of plant resources (biomass, nutrients, or palatability), but traditional techniques can only determine at most up to 8–12 and thus might not provide a comprehensive evaluation of diet composition in the natural grassland (Zhang, Jin, Badgery, & Tana, 2017). The observations herein showed that there were significant differences in herbage composition among the grazing patterns that resulted from the distinction of feeding regimes. The energy and albumen contents of concentrated pelleted feed in the time-limited grazing group were greater than in the forage roughage in the grazing group, and this influenced the plant groups consumed by sheep in the concentrate dominant group. Compared to the time-limited grazing group (grazing for 4 h), sheep grazing for 12 h were able to acquire more diverse plant species rather than relying primarily on *Lespedeza sp.* , and this can be attributed to their having sufficient time for choice. Regarding to the supplementary feeding group, *Lespedeza sp.* seemed to prevail due to its ubiquity as well as satisfactory palatability. In comparison, the grazing time in the time-limited grazing group was less than in the all-day continuous grazing group, resulting in less selectivity for plant species and higher transformation efficiency for forage.

It has been reported that the feedback for feeding behavior of sheep can be associated with the sensory quality traits of herbage taxa (Provenza, 1995). In the present study, the findings on the diet composition for the two periods showed a significant difference. The diet intake of sheep in the early grazing mainly consisted of *Lespedeza sp.* , but this changed to *Chenopodium sp.* , *Artemisia, sp.* , and*Lespedeza sp.* in the late grazing group. Tan sheep have been shown to forage largely on*Lespedeza sp.* , a common plant item in local desert steppe. Furthermore, metabarcoding diet analysis also demonstrated that Tan sheep indeed consume medicinal plants such as *Phellodendron amurense* , *Arnebia euchroma* ,*Ixeris tamagawaensis* , and *Convolvulus sp.* , consistent with other studies.

4.2 | Fatty acid deposition

This study represents our first effort to evaluate the correlations between herbage consumed and meat quality characteristics in sheep. Combining the dietary composition information with data on the deposition of muscle fatty acids has provided insights into how changes in dietary composition of grazing animals affect the fatty acid patterns in the *longissimus dorsi* . There was a significant alteration in the muscle fatty acid composition of the sheep as the overall diet community and biomass of plant taxa altered between the three treatments. Grazing herbivore preferences were largely dependent on the usability of plant items in the pasture. While the dietary structure or plant community of the habitat changed, feeding behavior preferences of sheep varied, and this variation might be the cause of different muscle fatty acid contents. Metabarcoding dietary analyses have provided a greater and more robust ecological context to decipher diet composition and dietary shifts.

The performance traits of grazing lambs are largely affected by nutrient components of the forage. Furthermore, the grazing feeding can promote the formation of beneficial fatty acids in sheep meat. Previous research has shown that the special flavor of lamb meat is probably mediated by short-chain fatty acids (SCFAs) and stearic acids. Compared to the control, the feeding patterns of pasture or supplementary feeding significantly improved the total n-3 PUFA in lamb meat, which has also been observed in other studies (Zhang, Jin, Badgery, & Tana, 2017). The deposition types of meat fat were further found to differ depending on the feeding regimes. In comparison with the control, a greater ($P < 0.001$) ALA proportion was detected in the meat of grazing lambs. ALA, as the precursor in the endogenous synthesis of EPA and DHA in animal tissues, plays a key role in the n-3 PUFAs synthesis in meat or milk (Van Elswyk, & McNeill, 2014). As expected, we found that the sheep meat derived from grazing lambs had remarkably greater proportions of EPA and DHA than the meat from the stall-fed group. It was reported that PUFAs accounted for a high ratio in the grass/forage (Figure S1) and that n-3 fatty acids were mainly synthesized in the plant chloroplasts (Watkins Jr, Mack, Sinclair, & Mulkey, 2007). Therefore, it is supposed that fatty acid profiles in lambs are associated with those of ingested herbage.

However, the deposition mechanisms of PUFAs in sheep meat are still not fully understood. As shown in Figure S1, multiple herbage taxa from field-collected plants contain plenty of PUFAs. Of those n-3 fatty acids, ALA presented a significant linear regression between the meat and dietary intake, a result that has been observed in previous studies. Comprehending the relevance of herbage ingested by sheep to the fat deposition is important for the production of high-quality meats and the regulation of meat products. The supplements of antioxidants such as PUFAs and lycopene in the diet have been shown to improve the quality of animal products. With the decreased proportion of corresponding herbage consumed by sheep, the percentage of saturated fatty acids decreased (Table 2). Therefore, we concluded that some herbage species can reduce the proportion of SFAs and consequently increase the proportion of PUFAs in the meat of sheep, thus realizing the improvement of mutton flavor. Nevertheless, the deposition of muscle fatty acids in the Tan sheep seems to be fairly insensitive to dominant herbage species such as *Lespedeza* and *Artemisia* (Figure 4 and Figure 5).

4.3 | Additional methodological considerations

Dietary metabarcoding techniques are being increasingly applied to investigate the diets of herbivores and omnivores, including the diets of pigeons, bears, monkeys, deer, zebras, and goats (Ando et al., 2013;

Quemere et al., 2013; De Barba et al., 2014; Kartzinel et al., 2015; Gebremedhin et al., 2016; Erickson et al., 2017). Much higher resolution is provided by the molecular analysis in comparison with the traditional alkane analysis of scats (Zhang, Jin, Badgery, & Tana, 2017). Both frequency of occurrence (FOO) data of food taxa and relative read abundance (RRA) of sequences are commonly used to convert sequence reads to dietary data. Both metrics have inherent biases when the number of food taxa in samples is large. In the current study, the diet data were presented using these two approaches to reduce any biases.

Accumulating evidence suggests that the number of sequence reads is approximately proportional to relative biomass of prey in a diet sample (Elbrecht, & Leese, 2015; Evans et al., 2016; Hänfling et al., 2016; Clarke, Beard, Swadling, & Deagle, 2017). However, the relationship between organism biomass and number of sequences can be variable due to PCR amplification bias of barcode primers (Albaina, Aguirre, Abad, Santos, & Estonba, 2016), and such biases were observed in our previous studies (Guo, Zhang, Chen, & Zhang, 2018). The utilization of different sets of barcode primers amplifying different gene regions could further improve the taxonomic resolution of the dietary items. Besides the consideration of primers, the prey taxa are important factors associated with the amplification bias. Our previous research showed that the sequencing process consistently overrepresented some herbage taxa (e.g., Leguminosae and Compositae) and underestimated others (e.g., Gramineae) relative to actual biomass. Nevertheless, a significant positive correlation between the DNA sequence proportion acquired from the sequencing procedure of feces samples and the actual biomass proportion in the diet was observed in this study, providing support for quantitative investigation using metabarcoding dietary analysis. Additionally, the determination of relative abundances of target items could be improved through post-sequencing quantification methods or correction factors (Matesanz et al., 2019).

Although metabarcoding is considered as a robust technique for assessing a species' presence and its relative abundance, there are some shortcomings. Some common Poaceae species (such as *Zea mays*) were missing from the diet of lambs in the time-limited group, thus being consistent with the findings from an earlier study (Guo, Zhang, Chen, & Zhang, 2018). The lack of certain food taxa may have resulted from methodological biases or from a draconian cutoff in the PCR protocols, as proposed by Vesterinen et al. (2016) and Clarke et al. (2014). In addition, incidental consumption of non-food DNA in the drinking water and fungi in the digestive tract can hinder the identification of certain groups. Furthermore, metabarcoding depends on single gene regions to detect food items, and this may cause that numerous related species to be indistinguishable (e.g., *Lespedeza potaninii* and*Lespedeza chinensis*). Having said this, the utilization of primer sets targeting composite barcodes (e.g., ITS2 and *trn* H-*psb* A) should be taken into consideration in future studies to further improve the taxonomic resolution and obtain a more thorough analysis (Pang, Shi, Song, Chen, & Chen, 2013). Likewise, establishment of reference databases targeting regional location-based items has been suggested as an important consideration of diet studies.

In conclusion, our results demonstrated that grazing systems could affect the fatty acid composition and promote n-3 PUFAs deposition in Tan sheep. Grazing and time-limited grazing feeding systems had lower ratios of n-6/n-3 fatty acids in the muscle than stall-fed modes, a result that was more in line with human health needs. Moreover, this research systematically studied the correlations between herbage taxa and omega-3 fatty acids and thereby has provided an initial view of the herbage effect on PUFAs of lambs. The significant correlations found in this study provide experimental evidence for future feeding research. The diet-PUFAs in the meat quality regulation of sheep may be one of the key research points for in-depth understanding of the flavor accumulation of beneficial health-related PUFAs.

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DATA ACCESSIBILITY STATEMENT

DNA sequencing data are submitted into the NCBI Sequence Read Archive (SRA) database with BioProject accession numbers PRJNA660588.

AUTHOR CONTRIBUTIONS

HLL, YPG, and XGZ conceived and designed the project; YPG performed samples collection, molecular work, bioinformatics, and statistical analysis; XGZ, ML, and CZ participated in the field trial; XGZ performed chemical analysis and provided fatty acid data; YPG wrote the manuscript; YJZ offered constructive suggestions on the grazing experiment; QM provided pasture and planning of experimental plots; BW and HLL contributed to writing. All authors have read and approved the final version of this manuscript.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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Tables

Table 1 Composition and nutrient level of experimental diet (percent dry matter basis).

Ingredients
TMR granules
Corn
Wheat bran
Rapeseed cake
Soybean meal
$NaHCO_3$
NaCl
Premix$^+$
Alfalfa hay
Hay
Alfalfa hay
Maize straw
Total

$^+$ The premix provides the following per kg diet: VA 320 IU, VD3 120 IU, VE 1000 IU, Cu 300 mg, Fe 500 mg, Zn 500 mg,

Table 2 Effect of different feeding regimes on fatty acid composition of the *longissimus dorsi* muscle of the Tan sheep lambs (% of total fatty acid).

Items	Group
	Grazing (G)
SFA	**SFA**
C10:0	0.15
C12:0	0.17
C13:0	**0.07**
C14:0	2.53
C15:0	**0.51**[a]

Items	Group
C16:0	**21.14[b]**
C17:0	1.32
C18:0	**20.97[a]**
C20:0	**0.2[a]**
C21:0	**0.72[a]**
C22:0	**0.24[a]**
C23:0	**0.20[a]**
C24:0	**0.21[a]**
MUFA	**MUFA**
C14:1	0.12
C16:1	1.26
C20:1	**0.17[b]**
C24:1	0.18
C18:1n9	**33.49[b]**
C22:1n9	0.07
PUFA	**PUFA**
C20:2	0.05
C18:3n3 (ALA)	**1.22[a]**
C20:5n3 (EPA)	**0.45[a]**
C22:6n3 (DHA)	**0.22[a]**
C18:2n6	9.41[b]
C20:3n6	**0.33[a]**
C20:4n6	**4.64[a]**
n-3 PUFA	**1.89[a]**
n-6 PUFA	**14.37[a]**
n-6/n-3	**7.69[c]**
[a, b] values swithin a row with different superscripts differ significantly at $P < 0.05$.	[a, b] values swithin a row with different

Table 3 Views of preferred diet items of the Tan sheep from different groups as interpreted from the relative read abundance (RRA) of the predominant ecologically significant OTUs in fecal samples.

Food items	**Sequence reads**	**Relative read abundance (RRA,%)**	**Relative read abundance (RR**
		G1	**G2**
Fabaceae			
Lespedeza sp.	**439080**	**31.27**	**12.90**
Medicago sativa	**47162**	**0.14**	**0.34**
Oxytropis halleri	**11557**	**3.20**	**0.02**
Astragalus peterae	7432	0.16	1.86
Caragana korshinskii	5967	0.54	0.31
Compositae			
Artemisia sp.	**232108**	**14.83**	**27.27**
Ixeris tamagawaensis	**17516**	**3.08**	**0.84**
Chenopodium			
Chenopodium sp.	**206047**	**1.77**	**39.34**
Corispermum sp.	**168798**	**9.46**	**0.10**
Salsola sp.	**44885**	**7.77**	**3.14**
Bassia scoparia	**35845**	**4.19**	**4.29**
Rutaceae			

Food items	**Sequence reads**	**Relative read abundance (RRA,%)**	**Relative read abundance (RR**
Phellodendron amurense	**56116**	**4.83**	**2.84**
Euphorbiaceae			
Euphorbia humifusa	**29279**	**6.29**	**0.86**
Boraginaceae			
Arnebia euchroma	**22751**	**4.12**	**1.67**
Poaceae			
Eragrostis sp.	21009	1.49	0.53
Setaria italica	11101	0.12	1.85
Chloris pilosa	9045	0.01	0.00
Cenchrus flaccidus	8715	1.80	0.18
Rosaceae			
Sibbaldianthe bifurca	15051	0.74	0.10
Convolvulaceae			
Convolvulus sp.	7986	0.86	0.22

Table 4 Variable score values from PCA and RDA.

Variable	**PCA**	**PCA**	**RDA**	**RDA**
	AX1	**AX2**	**AX1**	**AX2**
C18:2n6	-0.0916	-0.0066	-0.0389	-0.1015
C20:3n6	-0.1396	0.0629	0.0795	-01115
C20:4n6	-0.1908	0.2427	0.2962	-0.0896
C18:3n3 (ALA)	-0.2847	**0.5700**	**0.6590**	-0.0469
C20:5n3 (EPA)	-0.2838	**0.5497**	**0.6645**	-0.0470
C22:6n3 (DHA)	-0.2899	**0.5997**	**0.6722**	-0.0417
Lespedeza sp.	0.3985	-0.0145	-0.3333	0.2988
Artemisia sp.	**-0.8238**	0.2048	0.3445	**-0.7406**
Chenopodium sp.	**-0.7439**	0.0083	0.4847	**-0.6233**
Corispermum sp.	**0.9410**	0.1738	-0.1430	**0.9532**
Phellodendron amurense	-0.4417	0.2831	0.1155	-0.4098
Medicago sativa	0.3371	-0.3674	-0.4835	-0.5283
Salsola sp.	**0.8370**	-0.0721	**0.7182**	0.0605
Bassia scoparia	**0.7333**	0.0533	**0.6584**	-0.1157
Euphorbia humifusa	0.0208	**0.9539**	**0.7372**	0.3197
Arnebia euchroma	-0.0968	**0.9012**	**0.7549**	0.2081
Ixeris tamagawaensis	-0.1063	**0.6758**	**0.5390**	0.1010
Oxytropis halleri	0.2638	**0.8730**	**0.5869**	**0.5184**
Eigenvalue for the axis	0.3992	0.2273	0.2199	0.3769

Table S1 The relationships between polyunsaturated fatty acids and dominant diet items of sheep were analyzed using Pearson's correlation. Correlation coefficients are shown in the table. Significant correlations are noted with asterisks.

C18:2n6c
C20:3n6
C20:4n6

C18:3n3
C20:5n3
C22:6n3
Le
Ar
Ch
Co
Pa
Ms
Sa
Bs
Eh
Ae
It
Oh
Abbreviations are as follows: Lespedeza sp. (Le), Artemisia sp. (Ar), Chenopodium sp. (Ch), Corispermum sp. (Co), Phell

Figure Legends

Figure 1 Taxonomic relationships of the plants identified by DNA metabarcoding sequencing of the ITS2 gene in food residues of fecal samples. (a) Rarefaction curves of OTUs. (b) Venn diagram of shared OTUs between different samples. (c) PCoA plots based on Binary-Jaccard metrics between grazing patterns and grazing periods. PC1 is mainly associated with grazing group (left) or the time-limited grazing group (right). PC2 is mainly associated with the early grazing (bottom) or late grazing (top). (d) The heatmap of main plant composition for the Tan sheep in different grazing patterns.

Figure 2 Information in fecal samples from dietary metabarcoding data of sheep grazing on desert steppe grassland. (a) Individual-level data for 24 fecal samples described using different metrics. Colors indicate different plant taxa. (b) Population-level summarizes of the data showing the top 20 plant taxa. In both individual and population data, samples include diet items with >0.01% sequence reads.

Figure 3 The relative read abundances of major plant groups consumed by Tan sheep grazing in the desert steppe. Stacked bar charts show the top 20 plant taxa with 97.96% sequence reads. Colors represent different food taxa. Grazing types are grazing (G) and time-limited grazing (LG).

Figure 4 Principal component analysis (PCA) of diet items and polyunsaturated fatty acid variables in the axis 1 × axis 2 ordination planes, with all four of the treatments as an overlay (green diamonds and blue up-triangles).

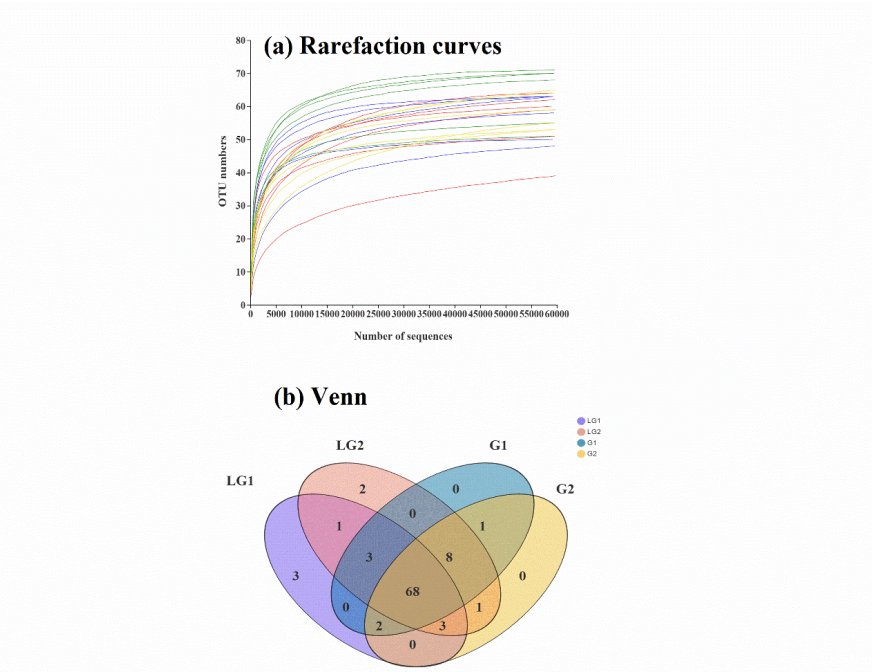
Figure 5 Redundancy analysis (RDA) of diet items and polyunsaturated fatty acid variables in the axis 1 × axis 2 ordination planes constrained by the two grazing treatments. The two treatments are: grazing (12 h) and time-limited grazing (4 h).

Figure 6 Pearson correlations between each PUFA and herbage taxa. Abbreviations are as follows: *Artemisia sp.* (Ar),*Corispermum sp.* (Co), *Phellodendron amurense* (Pa),*Medicago sativa* (Ms), *Lespedeza sp.* (Le),*Chenopodium sp* . (Ch), *Salsola sp.* (Sa), *Bassia scoparia* (Bs), *Euphorbia humifusa* (Eh), *Arnebia euchroma*(Ae), *Ixeris tamagawaensis* (It), *Oxytropis halleri* (Oh).

Figure S1 Fatty acid content in the herbage derived from the grazing habitat of Tan sheep.

Figures

Figure 1



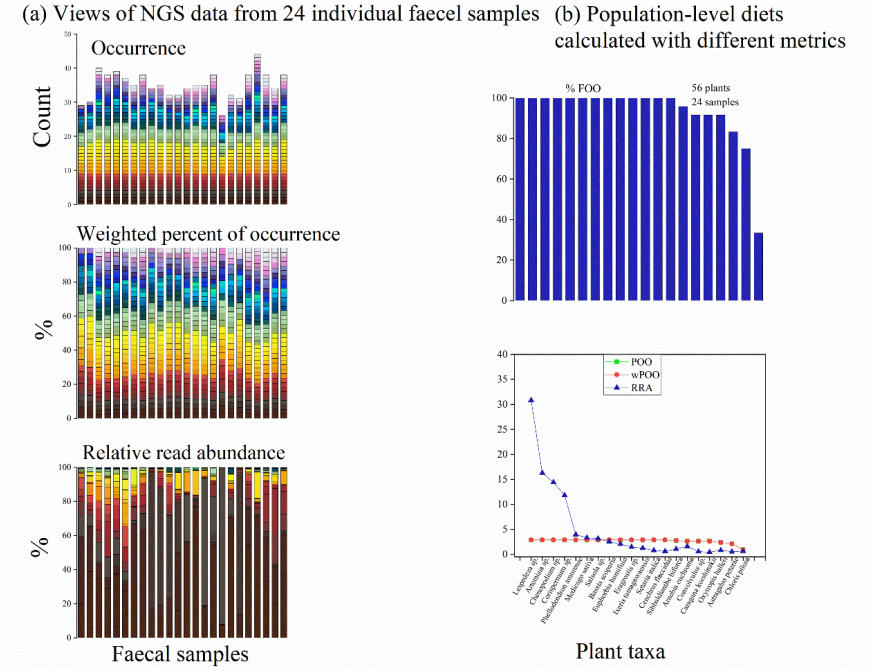
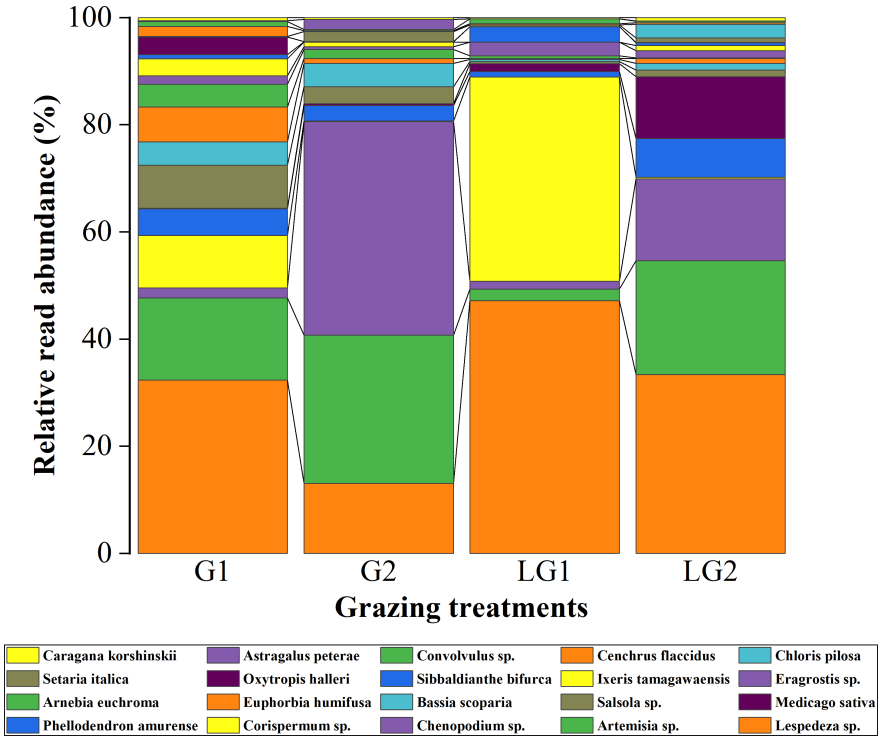


Figure 2

Figure 3



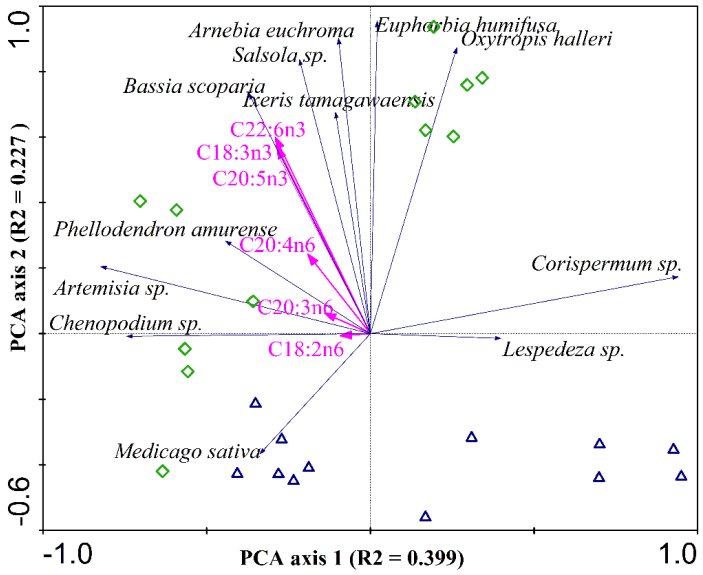


Figure 4

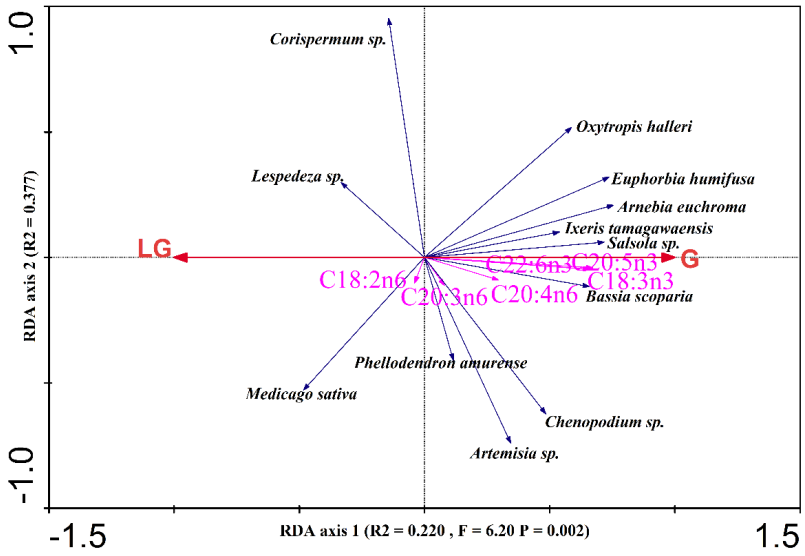


Figure 5

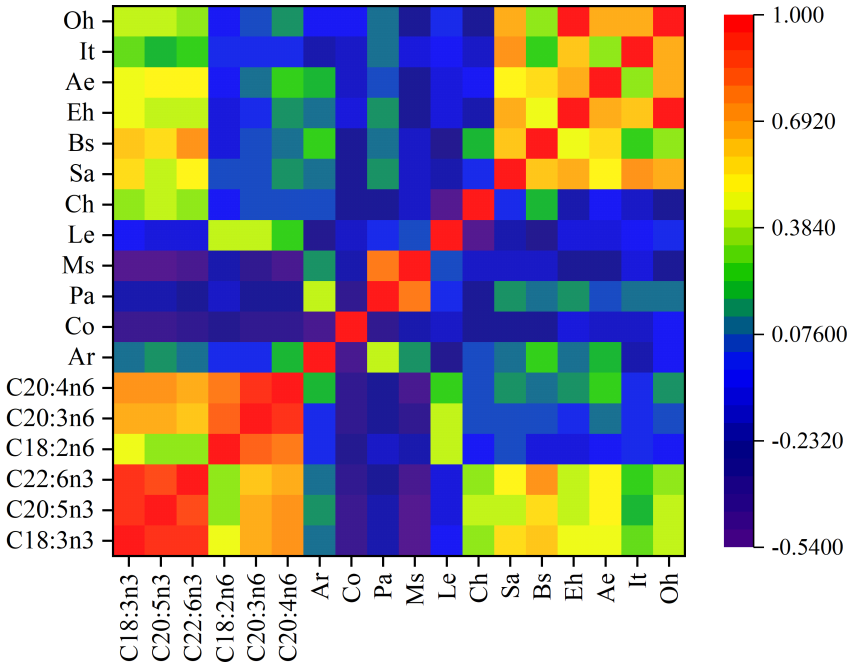


Figure 6

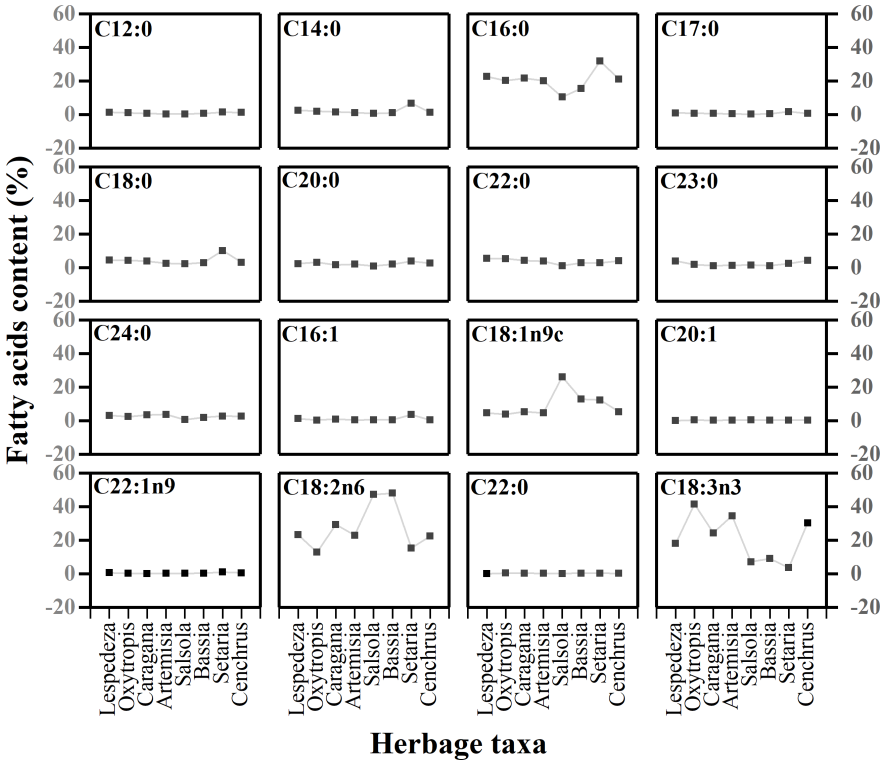
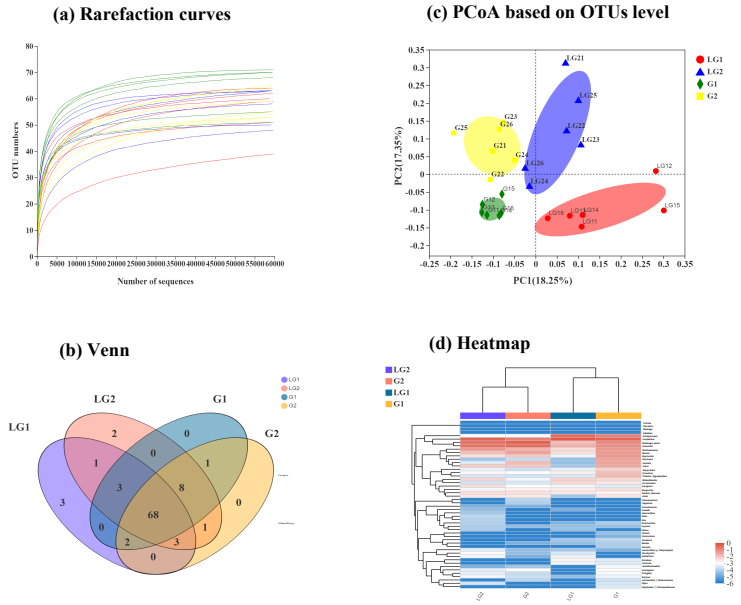
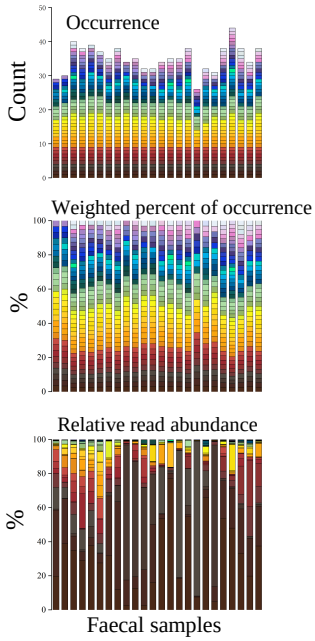


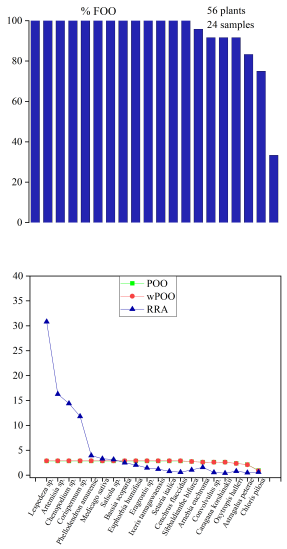
Figure S1

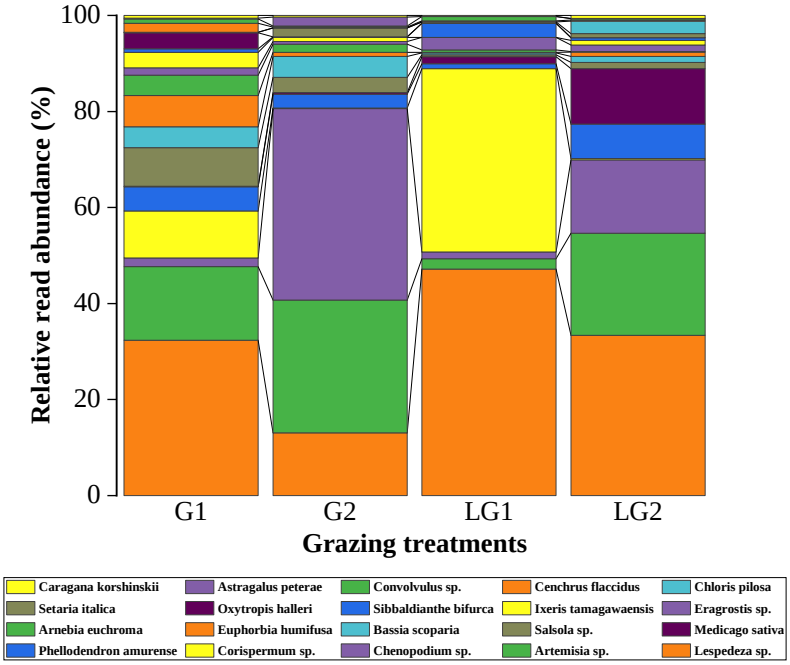


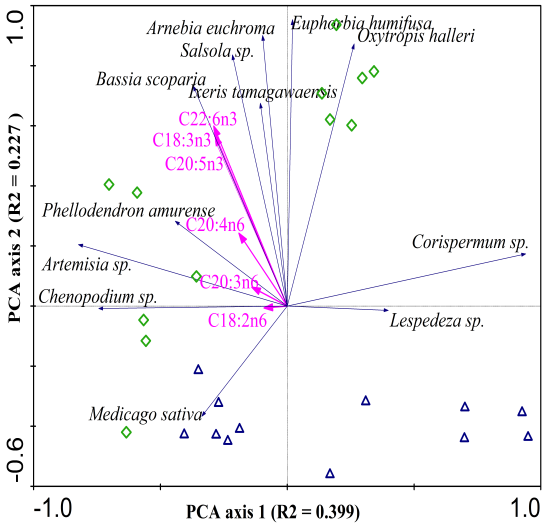
(a) Views of NGS data from 24 individual faecel samples

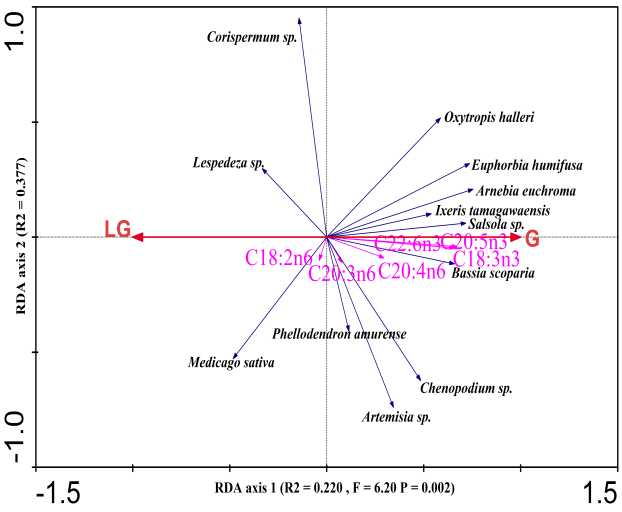


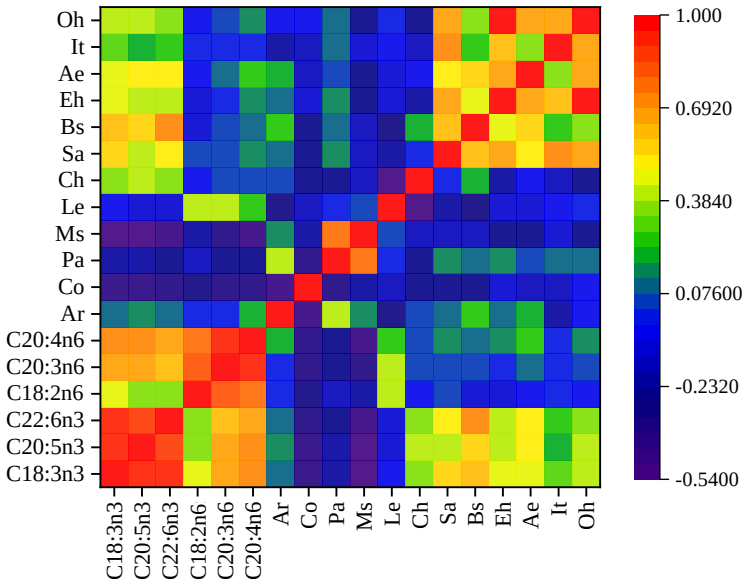
(b) Population-level diets calculated with different metrics

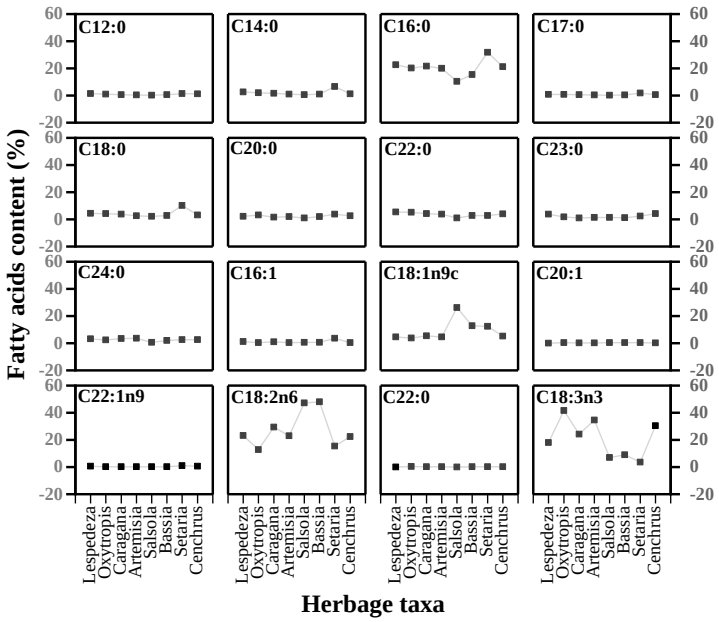












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`Table.docx` available at https://authorea.com/users/357096/articles/479827-association-between-dietary-components-and-muscle-fatty-acid-deposition-in-longissimus-dorsi-results-from-a-metabarcoding-diet-analysis-of-grazing-tan-sheep