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Validation of DNA metabarcoding of fecal samples using cattle fed known rations

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ABSTRACT

Diet composition of free-roaming animals has been persistently difficult to quantify. Fecal DNA (fDNA) metabarcoding is an emerging novel technology to reconstruct botanical composition of diets. Validation studies comparing fDNA metabarcodion to fed diets are lacking. A blind 6-week feeding study using 5 heifers (416–527 kg) fed different diets weekly with fecal samples collected from individuals 7 days after the introduction of each new diet was conducted. Diets were: (1) C3 grass hay (creeping meadow foxtail (*Alopecurus arundinaceus*)), (2) C4 grass hay (foxtail millet (*Setaria italica*), (3) C3 grass hay + C4 grass hay + alfalfa hay (*Medicago sativa*) (equal 1/3 proportions dry matter, 17.0%, 30.5%, and 52.5% by crude protein contribution, respectively), (4) C3 grass hay + C4 grass hay + alfalfa hay (equal proportions) + minor component of Wyoming big sagebrush (*Artemisia tridentata* ssp. *wyomingensis*) leaves (fed at 25 grams per heifer per day equal to 0.29% by dry matter contribution; crude protein content unknown), (5) alfalfa hay + minor components of crested wheatgrass (*Agropyron cristatum*), western wheatgrass (*Pascopyrum smithii*), and blue grama (*Bouteloua gracilis*) that included steams, leaves, and seed heads (varying proportions by dry matter ranging from 0.02 to 0.15% but 0.05%, 0.04%, and 0.01% by crude protein contribution), and (6) alfalfa hay. Single species diets were fed ad libitum. Diets with multiple forage species were fed at metabolic weight-based amounts with minor diet components introduced 2-3 days after major diet components. At the species level, fed diets and fDNA metabarcoding diets were dissimilar due to misidentification of major C3 and C4 diet components. For C3, *A. arundinaceus* was under the same Operational Taxonomic Unit (OTU) as *Phleum pratense* (not present in the C3 sourced hay), so blind identification was initially wrong. For C4, *S. italica* was identified as *Setaria palmifolia*, which does not grow in the region. Microscopic evaluation of seeds for both misidentifications verified the correct species identification and the fDNA error. Minor dietary components were detected in only 1 of 5 heifers for all

Abbreviations: BLAST, Basic Local Alignment Search Tool; C, Celsius; CI, confidence interval; C3, plant photosynthetic pathway where a 3-carbon molecule is the first produced and in grasses analogous with cool-season species; C4, plant photosynthetic pathway where an incoming carbon dioxide molecule attaches to the 3-carbon molecule producing oxaloacetate (a 4-carbon molecule) and in grasses analogous with warm-season species; d, days; fDNA, fecal DNA (deoxyribonucleic acid); fNIRS, fecal near-infrared reflectance spectroscopy; g, grams; IACUC, Institutional Animal Care and Use Committee; m, meters; MW, metabolic weight; OTU, Operational Taxonomic Unit; PCR, polymerase chain reaction; trnL, chloroplast gene region useful for identifying plant species; USDA, United States Department of Agriculture

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species. Increasing the utility of fDNA metabarcoding for accurately determining the diet composition of free-roaming animals will necessitate: 1) improving the reference library of voucher species specimens, 2) understanding by technical and scientific staff that multiple species may be attributed to the same OTU, and 3) greater verification of fDNA metabarcoding results using field-derived plant composition data, especially in rangeland settings where plant diversity can be intrinsically high.

1. Introduction

Quantifying diet composition of free-roaming livestock and wildlife can inform decision-making by producers to achieve desired objectives through predicting outcomes of grazing strategies and identifying key plant species to guide management (Holechek et al., 1982). Such information is also critical because livestock and wildlife consume different quantities of nutrients temporally as forage quality and animal nutritional needs asynchronously fluctuate (Scasta et al., 2016). While quantitative dietary information is known to be useful, diet composition of free-roaming animals in extensive and spatially heterogeneous environments has been persistently difficult to quantify (Garnick et al., 2018). This challenge persists because (1) animals can be problematic to locate spatially, (2) plant material differentially breaks down in ruminant and non-ruminant animals, and (3) identification of plant fragments is difficult, complex, and time consuming (Holechek et al., 1982).

Diet composition quantification has become increasingly sophisticated (Garnick et al., 2018). Early techniques used direct observation of animal bite counts (Sanders et al., 1980) with subsequent development of more invasive methods including surgical stomach analysis, and rumen and esophageal fistulation (Holechek et al., 1982). More recently, non-invasive post-digestive techniques such as fecal microhistology and near-infrared reflectance spectroscopy (fNIRS) technology has been used (Lyons and Stuth, 1992; Decruyenaere et al., 2015; Kartzinel et al., 2015; Ottavian et al., 2015; Núñez-Sánchez et al., 2016). Despite technological advances, individual techniques continue to have challenges and constraints (Garnick et al., 2018). For the bite count method, plant identification skills are required, animals must be spatially located, and only a single animal is observed at a time (Sanders et al., 1980). For surgical techniques, plant material may be masticated beyond recognition, sample size is small, methods are time intensive, and problems can arise from surgical procedures (Holechek et al., 1982). Constraints of fecal microhistology are that plant material in the feces may not be proportional to that consumed by the animal, trained observers are required, slow turn around time for sample analyses is slow, and bias and errors can be high (Holechek et al., 1982; Garnick et al., 2018). Accurately quantifying diet botanical composition at the species-level has been problematic for fNIRS (Garnick et al., 2018). In addition, many fNIRS studies only quantified a single plant species in the diet (Walker et al., 1998, 2002; Valiente et al., 2004) or very simple diets with only 3 or so plant species (Landau et al., 2004), although more recent attempts have developed fNIRS calibrations for complex diets for up to 25 species in the diet (Glasser et al., 2008).

Technological advances now permit reconstruction of diet botanical composition using fecal samples from free-roaming animals through the use of DNA (fDNA) metabarcoding using a single-locus identification system with implications for dietary protein composition (Valentini et al., 2009; Craine et al., 2015; Kartzinel et al., 2015). Advantages of this technique are similar to fNIRS with non-invasivity and large sample sizes, but determination of diet botanical composition at the species level is quicker given the automation of reading numerous plant DNA sequences at a single time compared to the need for individual fNIRS calibrations per plant species (Pompanon et al., 2012). fDNA metabarcoding has focused on the trnL intron located in the plant chloroplast using c-h primers and estimating species diet composition through associations to their plant species-specific dietary protein content (Craine et al., 2015; Kartzinel et al., 2015).

Recent fDNA applications have (1) enumerated dietary niche partitioning among African herbivores (Kartzinel et al., 2015), (2) identified seasonal and regional diet fluctuations for bison (*Bison bison*; Craine et al., 2015), and (3) quantified geographical patterns of cattle (*Bos taurus*) diets in North America (Craine et al., 2016). Validation of the fDNA metabarcoding technique is lacking through comparison of known fed diets to laboratory results. Previous work has generated notable surprises in diet botanical composition of free-roaming herbivores (Craine et al., 2015), including previously unknown and non-graminoid major diet components in cattle diets at a continental scale (Craine et al., 2016), and greater composition of non-graminoid plants in bison diets (Craine et al., 2015). In addition, identification of diet components with resolution only to the family or genus level in some cases has been identified as a limitation, even if additional primers were used (Garnick et al., 2018). Thus, many questions exist regarding more widespread use of fDNA in scientific and managerial applications of free-ranging animals. We therefore conducted a blind cattle feeding trial using known fed rations (Pompanon et al., 2012) with the objective to validate the use of fDNA metabarcoding. We asked: Does fDNA metabarcoding accurately identify known major and minor diet components?

2. Materials and methods

2.1. Animal management

Five non-gestating and non-lactating 2-year old Angus-cross heifers (weights ranging from 416 to 527 kg) were used in a 6-week feeding study. Animal care and use was approved under University of Wyoming IACUC protocol # 20170208DS00258-01. Heifers were penned individually in adjoining 160 m^2 (4.45 m × 36 m) well-drained pens that included shade, outdoor access, and automatic water availability.

2.2. Diet construction and feeding

A unique diet was fed each week over the 6-week study period and every heifer received every diet (February 7th through March 14th, 2017). Fecal samples from individual heifers were collected 7 d after each diet introduction. Homogeneous diets (1, 2, and 6; Table 1) were fed *ad libitum* and provided nutritional resources representing different photosynthetic pathways (C3 or C4) and/or plant growth types (grasses or legumes). Heterogeneous diets (3, 4, 5; Table 1) were fed at metabolic weight (MW) based rations using $MW^{0.75}$ based on individual heifer weights. These diets were comprised of relative proportions of the aforementioned components with or without minor components (Table 1). Prior to the start of the feeding trial, the heifers were being fed a mixture of the same C3 hay and legume hay as was subsequently used in the trials. Due to winter weather conditions, animal diets were adjusted on February 28th and March 1st to compensate for sub-freezing temperatures at −4 °C. Minor diet components were introduced 4–5 d before each fecal collection due to a limitation of plant material and wanting to get animals transitioned to main diet components. Samples from each forage source were analyzed for crude protein at a commercial lab using NIRS. Forage species information is provided in Table 2. Diets fed were: (1) C3 grass hay (creeping meadow foxtail (*Alopecurus arundinaceus* Poir.)), (2) C4 grass hay (foxtail millet (*Setaria italica* (L.) P. Beauv.)), (3) C3 grass hay + C4 grass hay + alfalfa hay (*Medicago sativa* (L.)) (equal 1/3 proportions by dry matter but 16.9%, 30.5%, and 52.5% by crude protein contribution), (4) C3 grass hay + C4 grass hay + alfalfa hay (equal proportions) + minor component of Wyoming big sagebrush (*Artemisia tridentata* Nutt. ssp. *wyomingensis* Beetle & Young) leaves (fed at 25 g per heifer per day equal to 0.29% by dry matter contribution; crude protein content unknown), (5) alfalfa hay + minor components of crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.), western wheatgrass (*Pascopyrum smithii* (Rydb.) Á. Löve), and blue grama (*Bouteloua gracilis* (Willd. ex Kunth) Lag. ex Griffiths) that included steams, leaves, and seed heads (varying proportions by dry matter ranging from 0.02% to 0.15% but 0.05%, 0.04%, and 0.01% by crude protein contribution respectively), and (6) alfalfa hay. Minor components were fed at 2.0 g per day for *B. gracilis* and 12.6 g per day for *A. cristatum*. Due to technician error, *P. smithii* was fed at 12.6 g beginning on day three, fed at 3.0 g on days four and five, and 6.0 g on day six with the exception of one heifer that was fed 4.6 g. Rationale for inclusion of such low trace component amounts was based on the rationale to determine if fDNA could detect low amounts in the diet. Nomenclature adheres to the USDA PLANTS database (https://plants.usda.gov; USDA NRCS, 2018).

2.3. Fecal collection and DNA metabarcoding analyses

Individual fecal samples collected on day seven of each diet were initially frozen; all frozen samples were then collectively thawed at the same time and subsampled for analyses at a commercial laboratory for DNA metabarcoding analysis. The fDNA metabarcoding process uses DNA extraction from fecal samples and then trnL c and h primers for PCR amplification. Clustered gene sequences are assigned an Operational Taxonomic Unit (OTU) identification number and matched to a reference library. The BLAST nucleotide program was used to identify unknown sequences using a 97% base pair matching minimum criteria (Craine et al., 2015, 2016).

2.4. Data analyses

fDNA metabarcoding results predicting dietary protein composition were organized into three different groups: (1) 'as fed', (2) 'laboratory non-discretionary', and (3) 'laboratory rectified'. The 'as fed' data represents the proportion of species in each fed diet that we expected relative to crude protein values and mass of feed consumed. The 'laboratory non-discretionary' data represents the proportion of species found in the dataset as reported directly by the laboratory, without technicians removing non-sensical OTUs. Each OTU was compared to a reference file and then checked against the USDA PLANTS database (USDA NRCS, 2018). Scientific names, genus, or family names were added to the dataset. OTUs with precise scientific names were merged and percentages relativized. The 'laboratory rectified' data group represents the proportion of species found in the dataset after a technician checked each OTU against a reference file and then checked each OTU against the USDA PLANTS database. If the species did not grow in the region from which the forage was sourced, the OTU was discarded. Species with similar OTUs were merged using the technician's botanical knowledge and fed rations information. We then assessed if plant species identified with fDNA metabarcoding were the

Table 1
Date of feeding trial initiation and diet composition to validation DNA metabarcoding of fecal samples for predicting plant species composition of cattle diets.

Start Date	[1]Fed Diet
February 7, 2017	1st - C3 Grass uniform (*ad libitum*)
February 14, 2017	2nd - C4 Grass uniform (*ad libitum*)
February 21, 2017	3rd - C3 + C4 + Legume (metabolic weight based ration)
February 28, 2017	4th - C3 + C4 + Legume + minor component of *Artemisia tridentata* leaves (metabolic weight based ration)
March 7, 2017	5th - Legume + minor components of *Agropyron cristatum* + *Pascopyrum smithii* + *Bouteloua gracilis* (metabolic weight based ration)
March 14, 2017	6th - Legume uniform (*ab libitum*)

[1] When only one source was used in the diet (1st, 2nd, and 6th diets) heifers were fed ad libitum or the ration was available at all times and the amount and frequency of intake was contingent upon the free choice of the heifer. Diets comprising multiple components were fed metabolic weight (MW) based rations using $MW^{0.75}$ based on each heifers initial weight.

Table 2
Forage species used to construct known diet combinations to validate DNA metabarcoding of fecal samples for predicting plant species composition of cattle diets.

[1]Photosynthetic Pathway, Annual/Perennial, Plant type	[2]Common Name	[2]Scientific Name	[3]Crude Protein @ Feeding
Major Diet Components			
C3, Perennial, Graminoid	creeping meadow foxtail	*Alopecurus arundinaceus* Poir.	5.3%
C4, Annual, Graminoid	foxtail millet	*Setaria italica* (L.) P. Beauv.	9.6%
C3, Perennial, Legume	alfalfa	*Medicago sativa* (L.)	16.5%
Minor Diet Components			
C3, Perennial, Shrub	Wyoming big sagebrush	*Artemisia tridentata* Nutt. ssp. *wyomingensis* Beetle & Young	N/A
C3, Perennial, Graminoid	crested wheatgrass	*Agropyron cristatum* (L.) Gaertn.	3.6%
C3, Perennial, Graminoid	western wheatgrass	*Pascopyrum smithii* (Rydb.) Á. Löve	5.2%
C4, Perennial, Graminoid	blue grama	*Bouteloua gracilis* (Willd. ex Kunth) Lag. ex Griffiths	5.3%

[1] C3 = cool-season and C4 = warm-season; also referred to as photosynthetic carbon metabolism (PCM).
[2] Nomenclature adheres to USDA PLANTS Database (https://plants.usda.gov/java/).
[3] Crude protein estimated using near infrared reflectance (NIR) spectrometry conducted using a Foss 2500 at wavelengths between 850 and 2500 nm to quantify presence of key forage quality factors based on NIRS Level 2 equations according to the NIRS Forage and Feed Testing Consortium.

same as what were fed (binary yes/no), proportion of minor plant species detections per heifer group (# out of five heifers) and relative % protein contributions across heifers.

3. Results

3.1. Can fecal DNA metabarcoding accurately identify known major diet components?

Fed diets and laboratory non-discretionary diet results differed due to misidentification of major C3 and C4 fed diet components with fDNA techniques. For the homogeneous C3 diet comprised of *A. arundinaceus,* the main species identified using fDNA techniques was timothy (*Phleum pratense* (L.)). This incorrect attribution was due to one OTU co-representing *P. pratense* and *A. arundinaceus*, along with several other *Alopecurus* species, at the 97% base pair matching level. Incorrect blind determination of the plant species clearly indicates local botanical knowledge is critical to correctly identify species when an OTU represents multiple species across multiple genera. We verified that *A. arundinceus* was the fed species in this diet using microscopic evaluation of seed characteristics from the hay source; thereby validating that *P. pratense* was an incorrect attribution to the OTU.

Following this correction, fDNA results for the pure *Alopecurus* (C3) diet identified six genera comprising > 1% of the diet, and an additional eight genera comprising 0.1–1% of the diet (Fig. 1a). Furthermore, fDNA significantly underestimated *Alopercurus/Phleum* as comprising 70.8% + 22.2% (mean + 95% CI) of the diet. At the functional group level, fDNA estimated C3 grasses comprised 84.6 + 24.9% (95% CI) of the diet, which was not different from the expected value of 100. Four of the five heifers were estimated to have > 90% C3 grass in their diet, but high variance around the mean was due to one heifer estimated to have 49% C3 grass and a substantial forb component (41% *Descurainia* spp.) in the diet.

For the pure *Setaria* (C4) fed diet, fDNA results correctly identified the genus, but incorrectly identified the species as *S. palmifolia* (J. Koenig) Stapf instead of *S. italica*. We verified, through microscopic evaluation of seed characteristics from the hay source, that the fed species was *S. italica*. Moreover, the hay seed source was confirmed as certified labeled Golden German foxtail millet (i.e., *S. italica*). *S. italica* was not identified at all from the fDNA results, although other *Setaria* species including *S. lachnea*, *S. parviflora*, *S. verticillata*, and *S. viridis* were identified at low levels (< 1%). It is concerning that the species identified by fDNA, *S. palmifolia*, originated in the Pacific islands and the only US states with suitable growing conditions are Hawaii, Texas, Louisiana, and Florida (not South Dakota where the hay was grown, purchased, and loaded by research staff; USDA NRCS, 2018; Wunderlin et al., 2018). For the pure *Setaria* diet, fDNA estimated five additional genera comprised > 1% of the diet and another 11 genera comprised 0.1–1% of the diet (Fig. 1b). Estimated dietary level for *Setaria* of 84.2% + 9.1% (mean + 95% CI) was significantly lower than the expected value of 100% (Fig. 1b). The largest source of error (*Medicago* at 4.3%) was a genus which the heifers had not consumed the week prior to the pure C4 trial, suggesting a greater than 7 d lag time in the rumen.

For the pure *M. sativa* (legume) diet, *M. sativa* was accurately identified by fDNA. Five other *Medicago* species were identified by fDNA at levels < 1%. The fDNA results estimated that *Medicago* comprised 82.5% + 20.2% (mean + 95% CI) of the diet, three other non-leguminous genera comprised > 1% of the diet, and another six genera comprised 0.1–1% of the diet (Fig. 2a and b). The largest source of error came from forb and shrub species, collectively estimated to comprise 11% of the diet, and C3 grasses, collectively estimated to comprise 6% of the diet.

When heifers were fed a mixed diet containing 17% C3 grass (*Alopercurus*), 30% C4 grass (*Setaria*) and 53% legume (*Medicago*) on a dietary protein basis, the fDNA method substantially underestimated the legume content at only 17.8% + 5.5% (mean + 95% CI) and overestimated both types of grass in the diet (Fig. 2c and d). The magnitude of the legume underestimation in this mixed diet was

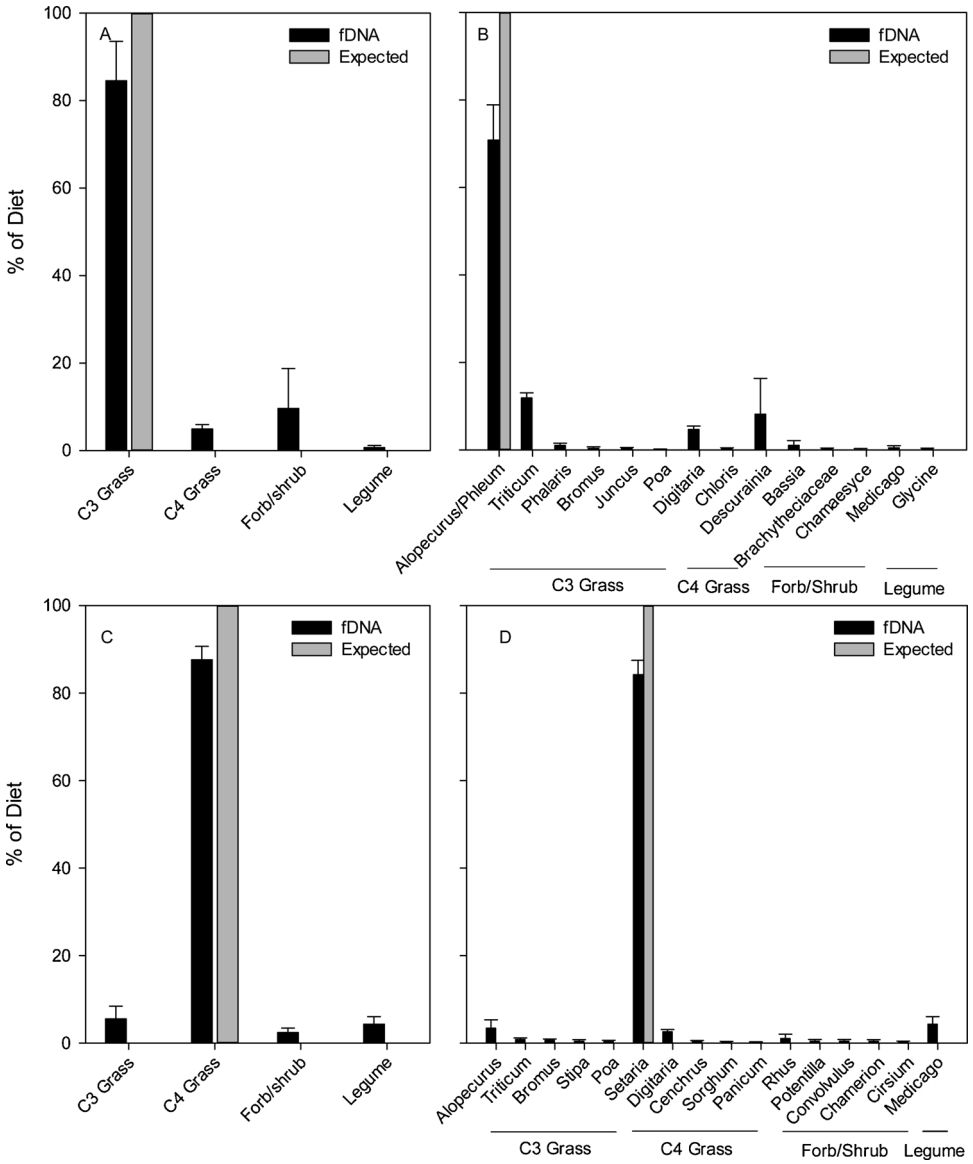

Fig. 1. Comparison of the expected (based on the fed diet) versus the measured (via fecal DNA metabarcoding) amounts of different plant functional groups (A,C) and genera (B,D) in the diet of five yearling heifers in feeding trials. Panels A and B depict results for heifers fed a pure diet of the C3 grass *Alopecurus arundinceus*. Panels C and D depict results for heifers fed a pure diet of the C4 grass *Setaria italica*. Error bars show 1 standard error calculated based on among-heifer variation.

greater than twice the level of underestimation when the legume was fed as a pure diet (Fig. 2b and d).

3.2. Can fecal DNA metabarcoding accurately detect known minor diet components?

The detection of minor components was variable. When heifers were fed a mixed C3/C4/legume diet that also contained a minor component of *Artemisia tridentata*, fDNA estimated that 0.7% + 1.9% (mean + 95% CI) of the diet was *A. tridentata*, close to the expected value of 0.4% based on an assumption of dry matter only as crude protein was unknown (Fig. 3). In addition, we note that results from this trial were very similar to results from the mixed-diet trial that did not include *A. tridentata*. In particular, dietary composition estimated by fDNA for the legume (19.5% + 10.5%) was substantially lower than the expected value of 53%, while composition estimated by fDNA for C3 and C4 grasses were greater than expected (Fig. 3).

In a separate trial addressing minor dietary components, heifers were fed a nearly pure *Medicago* diet that contained minor amounts of *A. cristatum*, *P. smithii*, and *B. gracilis* (expected to comprise 0.05%, 0.04%, and 0.01% of dietary protein content, respectively). *A. cristatum* was detected in one of five heifers and averaged across heifers, fDNA estimated *A. cristatum* was 0.02%, lower than the expected 0.05%. *P. smithii* was not detected by fDNA ever, however slender wheatgrass (*Elymus trachycaulus* (Link) Gould ex

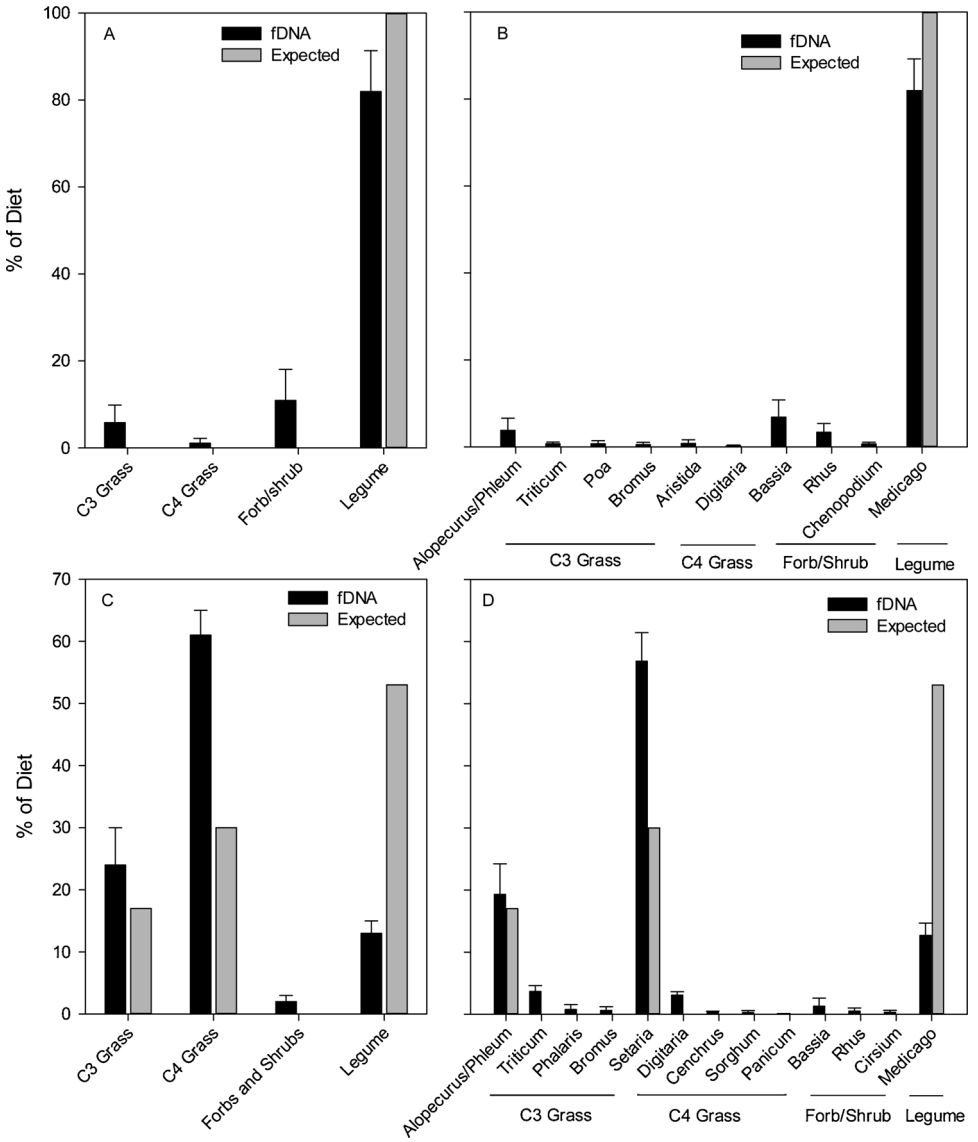

Fig. 2. Comparison of the expected (based on the fed diet) versus the measured (via fecal DNA metabarcoding) amounts of different plant functional groups (A,C) and genera (B,D) in the diet of five yearling heifers in feeding trials. Panels A and B depict results for heifers fed a pure diet of the legume *Medicago sativa*. Panels C and D depict results for heifers fed a mixed diet containing C3 grass (*Alopecurus arundinceus*), C4 grass (*Setaria italica*), and the legume *Medicago sativa*. Error bars show 1 standard error calculated based on among-heifer variation.

Shinners) was detected in one of five heifers. Given the phenological growth stage samples were collected, and because these are similar wheatgrasses that co-occur on the sampling site, it is possible that the two species were combined in the sampling effort. Averaged across heifers, fDNA estimated *E. trachycaulus* was 0.005%, lower than the expected 0.04%. *B. gracilis* was detected in one of five heifers and averaged across heifers, and fDNA estimated *B. gracilis* was 0.0009%, lower than the expected 0.01%.

4. Discussion

Our findings indicate several important limitations in the use of current fDNA methodology to quantify dietary composition of livestock. Specifically, fDNA results obtained and used without local knowledge of plant species in the sampled area or known diet (the case in our experiment) could lead to incorrect species level dietary compositions. It is possible to reach erroneous conclusions if fDNA is applied *carte blanche* in a manner similar to how more user-ready analyses of carbon, nitrogen, or crude protein quantification using other technologies are. This overarching caution is supported by our findings at the species level where fDNA was unable to correctly identify either the dominant C3 grass or the dominant C4 grass in our fed diets. Such misidentifications are concerning as Craine et al. (2015) suggested that *Beta vulgaris* may be important for range cattle diets in the southern US, yet *B. vulgaris* is known as

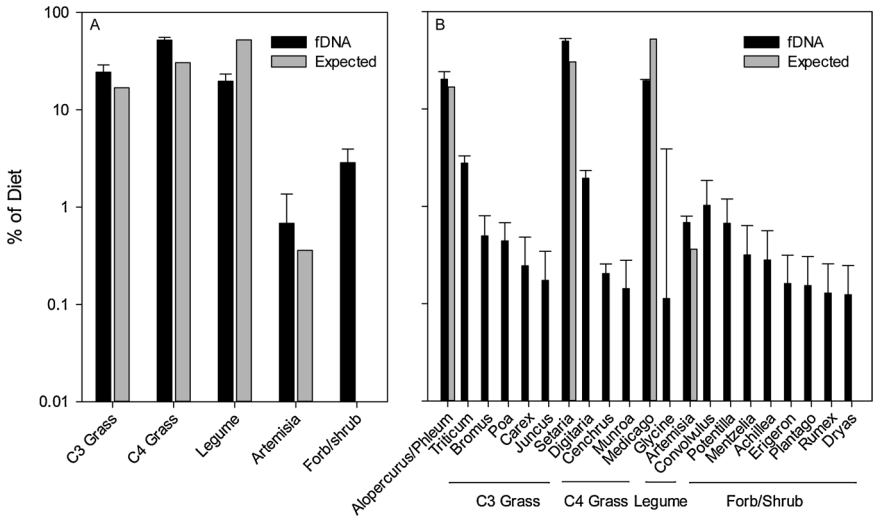

Fig. 3. Comparison of the expected (based on the fed diet) versus the measured (via fecal DNA metabarcoding) amounts of different plant functional groups (A) and genera (B) in the diet of five yearling heifers that received a mixed diet containing major components of C3 grass (*Alopecurus arundinceus*; 16.8%), C4 grass (*Setaria italica*; 30.5%), and a legume (*Medicago sativa*; 52.3%), and a minor component of the shrub *Artemisia tridentata* (0.36%). Note the logarithmic scale on the Y-axis. Error bars show 1 standard error.

common beets and is uncommon on North American rangeland and pasture (although it can be a feed additive). The authors of that study concluded that the OTU "best matched with *B. vulgaris* at the 97% identity level over 94% of the sequence, which suggests that the species that comprise the OTU are in the Amaranthaceae family, though might not be of the genus *Beta*" (Craine et al., 2015).

In addition to the uncertainty with fDNA at the species level, we observed additional uncertainty at the genera level. Prior efforts have indicated that identification at the family level may be the most accurate at times (Kartzinel et al., 2015). However, we were able to apply local botanical knowledge to infer which species could realistically be represented by the OTUs identified by the fDNA method, for both the C3 and the C4 grass. In addition, fDNA correctly identified the legume at the species level. Updated reference libraries that were made available after our initial experiment indicated that operational taxonomic units (OTUs) for *A. arundinaceus* and *P. pratense* were the same. Therefore, verification of fDNA results by knowledgeable technicians using plant composition data from the field is necessary in free-roaming rangeland and pasture applications, or any application including confined feeding experiments. In addition, if the laboratory had prior information about the key species on-site, that information could alleviate subjective interpretative decision making to correct species at the OTU level. To improve the use of fDNA metabarcoding to quantify protein diet composition of free roaming animals, it will be critical that knowledge of the plant community be available for reconciliation and verification of results. (Garnick et al., 2018).

Discrepancies in our study are attributed to fDNA misidentification based on the reference library available at that time and because fed diet sources were not homogeneous (i.e., hay meadows were likely more diverse than originally thought particularly for high proportion of *Descurainia* spp. (a weed in the mustard family that is common in hay may meadows dominated by C3 grasses) in the C3 hay diet). Yet some discrepancies remain unexplained including the mis-identification of *S. italica* as *S. palmifolia*. This suggests developing reference libraries for each study site using DNA sequenced plant voucher specimens could prevent problems (Valentini et al., 2009). This problem highlights the need for both users of fDNA technology and purveyors of the analyses to co-invest in producing DNA reference libraries that are much more exhaustive and encompassing. Future research should also test detection thresholds by incrementally increasing minor components in diets systematically.

Finally, our results show that the fDNA method can either significantly overestimate or underestimate the contribution of a given plant functional group to the diet, depending on what other functional groups are being consumed. For both C3 and C4 grasses, fDNA underestimated dietary contribution for animals fed pure diets (Fig. 1), but significantly overestimated their contribution when fed in combination with a highly digestible legume (Fig. 2c). The latter result suggests that differential digestibility of the dietary components may be an important source of bias in the fDNA method, just as others have shown for microhistological analyses of fecal material (McInnis et al., 1983; Mayes and Dove, 2000; Stewart et al., 2011). Correction of fDNA estimates based on estimates of the digestibility of dietary components at appropriate phenological stages may be necessary to achieve quantitative measures of diet composition.

5. Conclusions

fDNA metabarcoding is a novel technology with much potential for reconstruction of botanical composition of diets for free roaming animals, but caution must be emphasized when using this methodology. Our validation study suggests that the results can be incorrect and that three steps can help ensure more correct interpretation of the results. First, improving the content of the reference library to which OTUs are compared can enhance accuracy. Second, understanding that a single OTU can be attributed to multiple

species and interpretation must be georeferenced or compared to other sources of evidence. Third, fDNA metabarcoding results are optimal when verified with field-derived plant composition data, particularly in rangeland settings where plant diversity can be intrinsically high. Finally, using fDNA in tandem with other techniques such as microhistology could be powerful for both quality control and propelling fDNA for other applications and questions.

Animal care and welfare

Animal care and use was approved under University of Wyoming Institutional Animal Care and Use Committee (IACUC; Protocol # 20170208DS00258-01).

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Declaration of Competing Interest

After project completion, T.J. began working for Jonah Ventures, the laboratory that conducted the fDNA metabarcoding analyses. The authors declare no other conflicts of interest.

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