

The effects of increased hay-to-grain ratio on behavior, metabolic health measures, and fecal bacterial communities in four Masai giraffe (*Giraffa camelopardalis tippelskirchi*) at Cleveland Metroparks Zoo

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We evaluated whether increasing the hay-to-grain ratio offered to Masai giraffe (*Giraffa camelopardalis tippelskirchi*) at Cleveland Metroparks Zoo would reduce oral stereotypies and alter feeding behaviors, maintain or increase serum calcium-to-phosphorus ratio, decrease serum insulin-to-glucose ratio and salivary insulin, and alter fecal bacterial community structure. Giraffe transitioned to a ~90:10 hay-to-grain ratio in even increments over 8 weeks. A ration balancer was added during the seventh week of transition to ensure proper mineral and nutrient balance. We collected (1) behavioral data collected approximately daily using instantaneous focal sampling; (2) serum collections every other week for insulin-to-glucose and calcium-to-phosphorus ratio analysis and saliva weekly for insulin analysis; and (3) weekly fecal sample collections to examine changes in bacterial community structure during the 8 weeks preceding and following the diet change. After the diet change, giraffe spent significantly more time feeding and less time performing tongue and mouth stereotypies, people-directed and alert behaviors. Salivary and serum insulin and serum insulin-to-glucose ratio decreased, and fecal bacterial community structure changed significantly. However, serum calcium-to-phosphorus ratio remained >1:1 throughout the study. While further studies are needed to elucidate the nature and implications of the change in fecal bacterial community structure and metabolic hormones, the results of this study show promise for incremental improvements in health and welfare from feeding a higher proportion of forage in the diet.

KEYWORDS

insulin, ruminant nutrition, stereotypy, ungulates, welfare

1 | INTRODUCTION

Giraffe are iconic African animals, as well as one of the most popular and commonly exhibited species in zoos. More than 110 Masai giraffe

(*Giraffa camelopardalis tippelskirchi*) and 500 total giraffe live in AZA accredited North American zoos (ZIMS, Species 360 2017). Some zoo-housed giraffe experience behavioral and health issues, such as stereotypic behaviors, urolithiasis, and rumen health issues that may all

relate to diet. Therefore, changes in diet composition and structure may improve health and welfare.

Stereotypies in zoo-housed giraffe include pacing, tongue playing, inedible object licking, mane biting, and vacuum chewing (Seeber, Ciofalo, & Ganswindt, 2012). Bashaw, Tarou, Maki, and Maple (2001) reported that ~80% of zoo-housed giraffe performed at least one type of stereotypic behavior; the most prevalent was the licking of non-food objects. In the wild, giraffe use their tongues extensively while gathering and consuming browse. By contrast in zoos, giraffe may quickly consume their needed caloric and nutrient allotment if the majority of food is in the form of concentrates. Short feeding periods may leave them short of necessary amounts of oral stimulation (Baxter & Plowman, 2001), and contribute to the development of oral stereotypic behavior. Bashaw et al. (2001), suggested that oral stereotypies are more closely linked to feeding variables than to other environmental variables, such as enclosure size, and changes in diet have lowered stereotypic rates in other zoo species (Leeds et al., 2016; Less et al., 2014). For example, oral stereotypies were decreased and time spent ruminating increased with the addition of coarse meadow hay to giraffe diets (Baxter & Plowman, 2001). Therefore, diets that increase time spent feeding may beneficially influence giraffe behavior.

In addition to behavior, diet can influence health. Urolithiasis is a metabolic disease caused by the trauma or blockage of the urethra and bladder from uroliths, or urine crystals (Smith, 2009). This condition has been documented in zoo giraffe, with 10 of 41 zoos surveyed reporting conditions related to urolithiasis, and multiple known deaths due to urolith obstruction (Smith, 2009; Sullivan et al., 2010; Wolfe, Sladky, & Loomis, 2000). One possible cause of crystal formation is an imbalance in calcium-to-phosphorus ratio (Ca:P) in diet (Smith, 2009). While the recommended serum Ca:P is 1:1 or greater, ratios below one are often observed (Miller et al., 2010). Diets with higher hay-to-concentrate ratios produced a decrease in urine crystal count scores (considered a precursor to urolith formation) in meat goats (Sullivan et al., 2007). In a comparison of varying giraffe diets across five zoos, those with higher concentrate-to-hay ratios and thus, higher phosphorus levels tended to have higher serum phosphorus concentrations, which may result in greater risk for phosphatic uroliths (Sullivan et al., 2010). The rate of saliva secretion has been shown to be higher in cattle while feeding or ruminating than during rest (Bailey & Balch, 1961). Up to 80% of fecal excretion of endogenous phosphorus originates from saliva production (Bravo, Sauvant, Bogaert, & Meschy, 2003; Horst, 1986). Therefore, offering higher roughage amounts to increase feeding time and saliva production (Belknap & Pugh, 2002) may result in greater phosphorus excretion in feces (rather than urine) and lower risk of urine crystals.

Diet composition can also influence metabolism and body composition. Insulin, a hormone released from the pancreas, helps facilitate the movement of sugars into cells for storage or use (Brockman, 1978). For many mammals, low insulin and insulin-to-glucose ratios reflect greater insulin sensitivity and thus, better metabolic health (Guerrero-Romero & Rodriguez-Moran, 2001; Katz et al., 2000). Holstein cows fed a lower amount of concentrate had decreased glucose and insulin concentrations post prandial compared

to cows fed higher amounts of concentrate, which may reflect reduced insulin sensitivity in the cows fed higher amounts of concentrates (Jenny & Polan, 1975). Leptin, a hormone produced primarily by adipose tissue, is believed to play a central role in body energy homeostasis (Chilliard, Delavaud, & Bonnet, 2005), and has been used as an objective indicator in body condition in several species (Buff et al., 2002; Gamez-Vazquez et al., 2008; Sagawa, Nakadomo, Honjoh, Ishioka, & Saito, 2002). Leaner breeds in both beef and dairy cattle express lower plasma leptin levels with an exponential response of leptin to increased body fat (Chilliard et al., 2005). Thus, monitoring insulin-to-glucose ratios and leptin together can provide preliminary indications of the impact of a diet change on metabolism and body condition beyond simply monitoring weight.

Reduced rumination can also lead to changes in rumen bacteria and irritation with chronic rumen acidosis, D-lactic acidosis, or possible death (Smith, 2009). Clauss, Rose, Hummel, and Hatt (2006) observed rumen acidosis in half of giraffe where rumen pH was measured during post-mortem exams ($n = 4$). Excessive quantities of concentrate feed can increase the amount of acid producing organisms in the rumen by failing to stimulate proper rumination and saliva production (Owens, Secrist, Hill, & Gill, 1998). For example, both rumen and fecal pH decreased when ewes were fed high concentrate diets, indicating that concentrates may contribute to acidic conditions in the rumen (Neumann & Dehority, 2008).

Since saliva has qualities of a buffer and helps maintain acid-base equilibrium in the rumen (Gonzalez, Manteca, Calsamiglia, Schwartzkopf-Genswein, & Ferret, 2012), feeding a diet lower in concentrates may help stimulate saliva production, stabilize rumen pH, and favor a healthier rumen bacterial community. When examining the relationship between diet and bacterial communities, Neumann and Dehority (2008) found that both rumen and fecal total bacteria increased when animals were fed diets higher in concentrate compared to those higher in forage representing a potential link between the rumen and fecal bacterial communities. These demonstrated relationships between rumen and fecal bacterial populations indicate that changes in fecal bacteria may be an appropriate proxy for changes in rumen bacteria. Due to the difficulties in collecting rumen samples from zoo-housed ruminants, examining fecal bacterial communities may serve as a first step in examining the influence of diet changes in zoo-housed animals on gut bacteria.

In summary, there is an abundance of information to suggest that a high forage diet may have beneficial effects on behavior and overall health in zoo-housed giraffe. We hypothesized that a higher hay-to-grain ratio would reduce oral stereotypies while increasing time spent performing feeding behaviors, maintain or increase the serum Ca:P, decrease serum insulin-to-glucose ratios, maintain leptin levels (as a measure of body condition), and significantly alter fecal bacterial communities.

2 | METHODS

2.1 | Animals and animal care

Cleveland Metroparks Zoo (CMZ) housed three adult females, one adult male, and two calves during this study, though the calves were

not included in the study. One giraffe was in the first trimester of a 15-month pregnancy, and the two other females had calves of 6 and 5 months of age at the start of the 6-month study (Table 1). Both calves were consuming daily rations of both hay and concentrate (fed separately from the adults) in addition to occasional nursing. All animals were housed in a barn kept at approximately 21.5 °C throughout the study with access to a small outdoor runway only when the temperature exceeded 7.3 °C. Outside temperature averaged 0.6 ± 6.2 °C during baseline data collection and 6.9 ± 6.5 °C during post data collection (Weather Underground, 2018). The giraffe had access to the runway in 27% of baseline observations and 36% of post diet change observations, but chose to use the runway (not visible) in only 1.2% of observations. The length of daylight averaged 9.6 ± 0.4 hr during baseline data collection and 12.0 ± 0.7 hr during post data collection (Time and Date, 2018), but light cycles in the barn were kept constant throughout the study, with 12 hr light from 0600 to 1800 hours. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Cleveland State University and the Animal Care and Use Committee at CMZ.

2.2 | Experimental design

We aimed to change the form and volume of the feed offered without significantly altering nutrient content or caloric intake during the baseline and post diet change periods (Table 2). We provided browse (including but not limited to: Black Alder, Dogwood, Hawthorne, Honeysuckle, Pear, and Willow) intermittently, but consistently throughout the study and did not include this in nutrient analysis. Nutrient content was calculated based on information published by Mazuri Exotic Animal Nutrition (Richmond, IN) and either actual analysis of the alfalfa fed during the study (crude protein, crude fat, neutral detergent fiber, acid detergent fiber, lignin, starch, ash, calcium, magnesium, potassium, and digestible energy; completed by Dairy One, Ithaca, NY) or average alfalfa hay diet analysis (crude fiber, iron, sodium, zinc, copper, manganese, and selenium) reported by Dairy One. While we did not have target ranges other than to keep the nutrients as consistent as possible to the original diet, the recommendation of the giraffe nutrition workshop held by zoo nutritionists hosted at Lincoln Park Zoo in 2005 was to provide diets with between 10% and 14% protein, <5% starch, 2–5% Fat, 25–40% acid detergent fiber, 0.65–1.0% Calcium, 0.35–0.5% Phosphorus, 0.3% Manganese, and 10–15 ppm Copper (Schmidt & Barbiers, 2005). We gradually shifted the amount of each diet item (hay and grain) in evenly distributed weekly increments over a period of 8 weeks. The male

transitioned from an existing 50:50 diet ratio of 6.81 kg alfalfa hay and 6.81 kg Mazuri Wild Herbivore (Mazuri Exotic Animal Nutrition, Richmond, IN) pelleted grain daily to a ~90:10 ratio of 11.9 kg of hay and 1.4 kg of grain per day. The females, who were group fed, transitioned from a 70:30 baseline ratio of 9.1 kg alfalfa hay daily and ~3.7 kg Mazuri Wild Herbivore pelleted grain per female to a ~90:10 ratio of 11.9 kg hay and 1 kg of grain per female per day (Table 3). We recorded percent daily food consumption and weekly giraffe weights to ensure weight remained stable. Beginning the seventh week of diet transition onward, we offered a ration balancer (0.53 kg/female, 0.8 kg/male daily; Tribute Alfa Essentials, Upper Sandusky, OH) to ensure diets were meeting daily vitamin and mineral requirements.

2.3 | Behavioral data collection

We collected behavioral data from November 16, 2014 to April 29, 2015 with 58 observation periods per animal during baseline and 64 per animal for the post-diet change. We conducted observations approximately once daily, distributed evenly into six, 90-min periods (0800–0930, 0930–1100, 1100–1230, 1230–1400, 1400–1530, 1530–1700) throughout the week. Baseline and post observations were balanced for time of day and day of the week. Before the study began, giraffe were habituated to the presence of an observer in the barn over a period of several weeks. Baseline data collection began when giraffe started to spend the majority of time in their indoor enclosure due to declining temperatures. Volunteers collected data using an exhaustive ethogram that was mutually exclusive within each of two channels (Table 4). The two channels consisted of (1) feeding related and (2) all other recorded behaviors, such that two behaviors were marked at each observation point. Behaviors in each channel were prioritized from top to bottom of the listed ethogram such that only a single behavior was recorded on the beep within each channel. It should be noted that we recorded two forms of oral stereotypic behaviors, licking, and other tongue/mouth stereotypies to compare to previous studies in giraffe where they have been separately defined (Bashaw et al., 2001; Veasey, Waran, & Young, 1996), and only one of the two (tongue/mouth) reported in free-ranging giraffe. Ten observers were trained, and all achieved >90% inter-rater reliability with the primary investigator. We collected 80-min observations using randomly created starting orders of giraffe to be observed (www.random.org/list). We completed 5 min of focal follow per animal with instantaneous scan sampling at 1-min intervals, where the behavior was recorded according to the behaviors outlined by the ethogram. A total of four, 5-min focal follow sets were completed per 80-min observation so that each animal was observed for a total of 20 min per observation. For each observation, we noted the observer and whether the giraffe had access to the outdoor run.

2.4 | Biomaterials collection

Serum, saliva, and fecal samples were all individually labeled with date and animal ID and stored frozen in a –20 °C manual defrost freezer until analysis. We collected all serum and saliva before the first

TABLE 1 Sex, age, and maternal status for each giraffe

Giraffe	Symbol	Sex	Age	Status
A	Δ	Female	8	Intermittent nursing
B	○	Female	7	1st trimester pregnant
C	◇	Male	8	NA
D	□	Female	6	Intermittent nursing

TABLE 2 Nutrient composition estimates on a dry matter basis of female and male giraffe diets before (baseline) and after (post) an increase in the hay-to-grain ratio based on manufacturer published nutritional content of grain and ration balancer, and averaged nutrient analysis for alfalfa hay batches fed during the study

Nutrient	Females baseline	Females post	Male baseline	Male post
Crude protein (%)	14.05	14.58	14.24	14.40
Crude fat (%)	2.61	2.63	2.89	2.73
Crude fiber (%)	29.22	28.30	31.19	28.01
NDF (%)	52.69	52.24	55.28	51.65
ADF (%)	37.88	38.03	38.92	37.26
Lignin (%)	6.82	7.52	6.35	7.25
Starch (%)	2.24	2.12	2.89	2.44
Ash (%)	8.01	8.35	8.27	8.38
Calcium (%)	0.82	0.81	0.89	0.83
Iron (%)	0.04	0.04	0.04	0.04
Magnesium (%)	0.32	0.32	0.34	0.33
Phosphorus (%)	0.24	0.31	0.26	0.34
Potassium (%)	1.85	1.99	1.75	1.92
Sodium (%)	0.25	0.24	0.33	0.27
Zinc (mg/kg)	52.23	45.58	76.24	57.44
Copper (mg/kg)	11.81	16.76	15.04	21.23
Manganese (mg/kg)	64.99	53.87	87.36	61.62
Selenium (mg/kg)	0.25	0.77	0.33	1.09
DE (kcal)	34,492	34,656	37,943	37,835

NDF, neutral detergent fiber; ADF, acid detergent fiber. DE, estimated digestible energy as reported by the feed manufacturer or Dairy One analysis.

morning feed and collected whole blood approximately every 2 weeks by venipuncture in animals conditioned for voluntary blood draws. We isolated serum from whole blood per routine protocols and placed serum into a polypropylene tube labeled with animal ID, and date collected. We collected saliva weekly by stationing the giraffe voluntarily at the side of their enclosure and offering a 4-inch synthetic salivette (Salimetrics, Carlsbad, CA) to manipulate until saturated with saliva. The sample was then placed in the supplied 15 ml tube with collection chamber (Salimetrics). Immediately prior to

analysis, we thawed the saliva at room temperature for 20 min and centrifuged (2500g, 15 min) in supplied 15 ml tubes with collection chamber (Salimetrics). Fecal samples (approximately 50 g) were collected weekly into sealed, sterile plastic bags.

2.5 | Serum and saliva analysis

We validated assays to measure metabolic salivary and serum biomarkers in giraffe in conjunction with another study (Long, Raghanti, & Dennis, 2010).

TABLE 3 Outline of diet change

	Timeline	Baseline								Diet change								Post							
		Nov				Dec				Jan				Feb				Mar				Apr			
		Week	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7
Females	Hay (kg)	27.3																35.7							
Females	Grain (kg)	11.4																2.8							
Male	Hay (kg)	6.8																11.9							
Male	Grain (kg)	6.8																1.4							
Ration Balancer 0.5 kg per female 0.8 kg male																									

Female amounts given on a group fed basis ($n = 3$ females).

TABLE 4 Ethogram of behaviors used in study

Channel 1 Behaviors
Feeding
Hay—Subject is putting hay in mouth or chewing in close proximity to hay trough when hay is present
Concentrate—Subject is putting grain in mouth or chewing in close proximity to grain feeder when grain is present
Browse—Subject is putting browse in mouth or chewing in close proximity to provided browse when browse is present
Chew—Subject is chewing without evidence of what is in mouth in a consistent circular motion. Proxy for ruminating.
Drinking—Subject is drinking water
Other—Subject is consuming another substance not listed above such as lettuce or salt block. Please note what is being consumed
Not feeding—Subject is not performing a feeding behavior
Channel 2 Behaviors
Undesirable
Pace—Subject is moving around enclosure for no apparent goal or reason following an almost identical path for more than two repetitions
Tongue/mouth—Subject is moving tongue outside of mouth without apparent reason for longer than 2 s or moving mouth/chewing in an erratic fashion.
Lick—Subject is seen repetitively (>3×) licking or mouthing non-edible items
Social
Agonistic—Aggressive Behaviors: threat, leaning, sparring. Recorded when subject is initiator.
Affiliative—Behaviors: Nosing, licking briefly, rubbing, nursing. Recorded when subject is initiator.
Movement
Locomotion—Movement of subject that results in change of location by more than one body length
Stationary—Subject is standing or laying and eyes either open or closed, not seemingly focused at a particular point and/or object.
Alert—Standing or laying still with eyes open and focused on one point.
Directed
Self-directed—Subject is investigating, or touching self, or scratching on an object.
People-directed—Animal is clearly focused on or interacting with a human.
Object-directed—Investigating, touching or manipulating an object
Other
Animal is performing a behavior not described by any other category. Please note the behavior.
Not visible

We quantified insulin using a commercial bovine insulin enzyme immunoassay (EIA; Mercodia, Winston Salem, NC) according to the manufacturer's instructions. Serum and saliva serially diluted with supplied assay buffer demonstrated parallelism with the standard curve ($t = -0.016$, $p = 0.98$ serum, $t = 2.44$, $p = 0.47$ saliva). Recovery of samples spiked with known amounts of standard curve averaged 88% and 97%, for serum and saliva, respectively. Assay sensitivity was 0.03 ng/ml and intra- and inter-assay coefficients of variation for saliva were 4.83% and 2.46%, respectively. For serum insulin, we included all samples within a single assay, and the intra-assay CV was 3.84%. We biologically validated Insulin by demonstrating significantly higher concentrations in non-fasted compared to fasted samples (Paired *T*-test; $t = 3.384$, $p < 0.01$). We measured serum leptin using a commercial EIA with the provided Mouse standards (B-Bridge International, Inc, Santa Clara, CA). Serially-diluted serum exhibited parallelism with the standard curve ($t = -0.20$, $p > 0.05$). Recovery of samples spiked with known amounts of standard curve averaged 93% and assay sensitivity was 100 pg/mL. We included all samples within a single

assay and the intra-assay coefficient of variation was 2.96%. We quantified serum calcium, phosphorus, and glucose using a standard serum chemistry analyzer (Vet Test 8008; IDEXX Laboratories, Inc., Westbrook, ME).

2.6 | Fecal bacteria DNA extraction and purification

We extracted DNA using a bead beating extraction protocol (Burke, Dunham, & Kretzer, 2008). In brief, we added fecal material (0.25 g) to a bead beating tube with sterile forceps along with 700 μ l of cetyltrimethyl ammonium bromide (CTAB) and put on a Precellys 24 Homogenizer (Bretin Technologies, Montigny-le Bretonneux, France) for 40 s. We completed extract purification using phenol/chloroform procedure followed by precipitation in polyethylene glycol 8000 in 2.5 M NaCl (approximately 150 μ l). We then resuspended nucleic acids in 100 μ l of Tris-EDTA buffer, and transferred the solution to siliconized tubes to store at -20°C until analysis.

2.7 | Polymerase chain reaction-terminal restriction fragment length polymorphism (PCR-TRFLP)

The primer set 338f and 926r (Muyzer, de Waal, & Uitterlinden, 1993; Muyzer, Teske, & Wirsén, 1995) amplified the 16s rRNA gene region of the bacterial DNA following Burke et al. (2008). The reverse primer was labelled with 6-carboxyfluorescein (6FAM) and the forward primer labelled with 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein (HEX). We performed PCR in 50 µl reactions with 35 rounds of amplification in a PTC-200 thermocycler (GMI, Ramsey, MN) as described previously (Burke et al., 2008). Labelled amplicon product generated at Holden Arboretum using the endonuclease *Mbol* were sent to the Life Sciences Core Laboratories Center (Cornell University) where TRFLP profiles were created. These were then analyzed using GS600LIZ size standards and Peak Scanner™ Software (Version 2.0, Applied Biosystems, Foster City, CA).

2.8 | Behavior and hormone statistical analysis

Time spent chewing was used as a proxy for rumination since rumination could not be reliably observed in the barn. We combined time spent feeding on hay, concentrate, and browse as well as time spent chewing into a single category called feeding. We analyzed all behaviors using a generalized linear mixed model (PROC GLIMMIX; SAS Institute, Cary, NC) with individual animal included as a random factor. We included a random slope when it contributed to a better model fit as follows; day of week for people-directed and alert behavior models, and time of day for pacing, feeding, object-directed, self-directed, and stationary behavior models. We used a negative binomial distribution with a log link function for all behaviors with an offset for time spent visible. We used the between/within method to calculate degrees of freedom and present all behavior data as % of time visible.

We log transformed serum insulin, glucose, insulin-to-glucose ratio (a proxy for insulin sensitivity), leptin, calcium, phosphorus, and Ca:P as well as salivary insulin concentrations to approximate a normal distribution and then analyzed the data using a general linear mixed model (PROC MIXED; SAS Institute, Cary, NC). We used the same random factor and method for calculated degrees of freedom as described for behavior analysis. We built models using maximum likelihood estimation and computed final models using restricted maximum likelihood (REML).

For both PROC GLIMMIX and PROC MIXED, we chose models with the lowest -2 log likelihood estimates as the final model. We always included treatment (Baseline vs. Post diet change) in the model regardless of whether it was significant because it related to the main hypothesis. We removed all other factors from the model at $p > 0.10$ unless they contributed to a significantly lower -2 log likelihood. We present Post-hoc results as the least squared means \pm SEM. We analyzed giraffe weight from baseline and post diet change using a paired *t*-test with all baseline and post diet change weights for each giraffe averaged and paired for the analysis.

2.9 | TRFLP statistical analysis

TRFLP profiles were processed using the TRFLPR package in R (Petersen et al., 2015; R Core Team, 2014). Only peaks which accounted for greater than 1% of the relative peak area were included in sample analyses. TRFLP profiles were arcsine-square root transformed prior to analysis. Nonmetric multi-dimensional scaling analysis (NMDS) was used to assess bacterial community structure across the treatment (before vs. after diet change) using the metaMDS function in the Vegan package in R (Oksanen et al., 2007; R Core Team, 2014). The number of dimensions was selected using a scree plot and distances were determined using the Bray-Curtis method. Differences in centroid location between treatment and animal were tested via PERMANOVA using the adonis function in the Vegan package (Oksanen et al., 2007). For all statistical analyses, $p < 0.05$ was considered statistically significant.

3 | RESULTS

All giraffe finished their hay on $>80\%$ of feedings. On days when they left a portion of their hay, the average amount left over averaged less than 6% (range 3.7–29.4%) of total hay intake during both baseline and post-treatment. Giraffe consumed all concentrate every day throughout the study. Giraffe weight did not change significantly from baseline to post diet change (Paired *T*-Test; $T_{(3)} = -0.26$, $p = 0.81$; Figure 1).

3.1 | Behavior

Increased hay-to-grain ratio fed to Masai giraffe at CMZ correlated to a significant increase in the percent of time spent performing feeding behaviors ($F_{(1,3)} = 15.4$, $p = 0.03$; Figure 2A), and time stationary ($F_{(1,3)} = 30.9$, $p = 0.01$; Figure 2a), with increases in both behaviors in all four giraffe. The amount of time spent performing tongue and mouth stereotypies decreased by approximately 50% post diet change (4.3 ± 2.3 baseline vs. 2.1 ± 1.1 post % time; $F_{(1,3)} = 11.60$, $p = 0.04$; Figure 2b), with decreases also observed in all four giraffe. People-directed ($F_{(1,3)} = 34.63$, $p = 0.01$; Figure 2b) and alert behaviors ($F_{(1,3)} = 17.97$, $p = 0.02$; Figure 2b) also decreased following the diet change in all four giraffe. One giraffe (B) spent close to twice as much time performing alert (7.9%) and people-directed behaviors (10.10%) compared to averages for the other giraffe (4.46%, 6.04%, respectively) during the baseline period. Following the diet change, the amount of time spent performing these behaviors was more comparable to others (2.5% of time alert giraffe B vs. 1.2% average of other giraffe, 2.0% of time people-directed for giraffe B vs. 2.0% average of other giraffe, respectively). Hay-to-grain ratio was not correlated to time spent chewing, licking, pacing, locomotion, social, object- or self-directed behaviors. After time spent stationary and feeding, locomotion was the most common behavior. Licking stereotypies and pacing did not change, continuing at an average of 5.0% and 1.8% of time, respectively. All other behaviors in the ethogram were observed less than 1% of time. Giraffe were not visible for an average of 0.9% during the baseline and 1.5% during the post

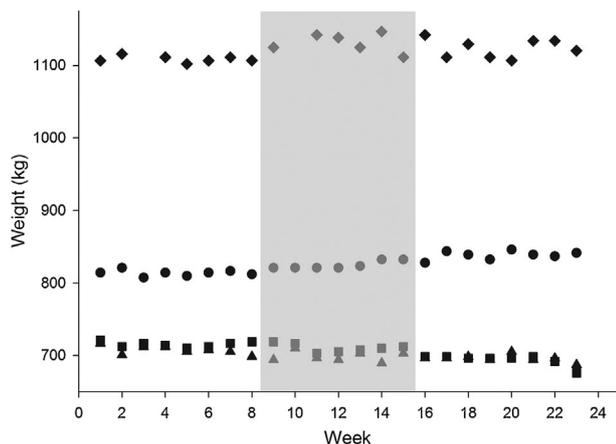


FIGURE 1 Giraffe weights from baseline to post data collection. Individual animals a, b, c, and d are represented by triangles, circles, diamonds, and squares, respectively (see Table 1). Pre- and post-diet change are represented by the first and second grouping of black markings on a white background, respectively while markings on a gray background represent the transition period weights. Note that the Y-axis does not start at zero to better visualize giraffe weight over time

period. Neither the observer, nor whether the giraffe had access to the outdoor run on a given observation were significant factors in any of the behaviors observed.

3.2 | Physiological changes

Increasing the hay-to-grain ratio fed to Masai giraffe at CMZ correlated to a decrease in average serum ($F_{(3)} = 24.71, p = 0.02$) and salivary insulin ($F_{(3)} = 16.56, p = 0.03$) concentrations, serum glucose concentrations ($F_{(1,3)} = 12.91, p = 0.04$), and serum insulin-to-glucose ratios ($F_{(3)} = 10.47, p = 0.048$) in all animals. Least squared mean salivary and serum concentrations before and after the diet change are depicted in Table 5. Serum leptin also decreased significantly after the diet change ($F_{(3)} = 15.01, p = 0.03$). However, on further post-hoc analysis, this difference was due to a single individual, Giraffe B who was in the first trimester of pregnancy, while leptin remained consistent in all other individuals throughout the study. Leptin in Giraffe B was the lowest of all four giraffe during both pre- and post-diet change periods. It is also interesting to note that the male giraffe had approximately three-fold higher average serum insulin concentrations (26.95 uIU/ml) and at least three-fold higher average serum leptin concentrations (880.72 ng/ml) compared to the individual averages for the three females (insulin 7.95, 13.07, and 7.15 uIU/ml; leptin 254.77, 59.69, and 85.95 ng/ml, respectively). The male also experienced a more dramatic and consistent decrease in salivary insulin post diet change (Figure 3c), compared to the females (Figures 3a, 3b, and 3d). However, all four animals experienced a decrease in salivary and serum insulin, and a progressive decrease in insulin-to-glucose ratio (Figure 4) throughout the study. The diet change did not correlate to serum blood calcium levels, phosphorus levels, or Ca:P (Table 5).

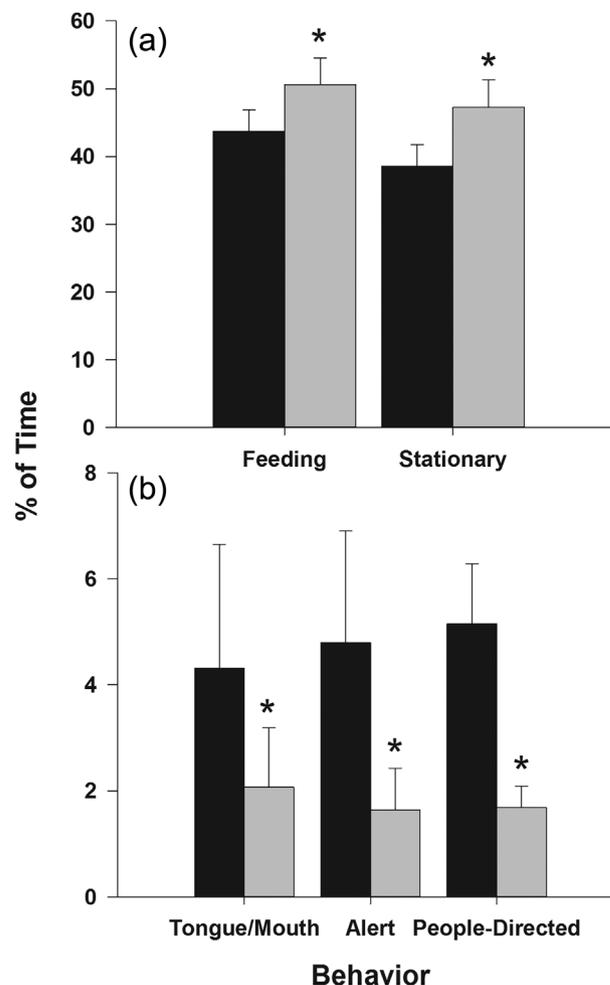


FIGURE 2 Least squared mean \pm standard error of the mean (LSM \pm SEM) % of time spent performing (a) feeding and resting, and (b) tongue and mouth stereotypies, alert and people-direct behaviors during the baseline (black bars) and post (gray bars) diet change periods. Note that feeding behaviors were analyzed independently of the other behaviors. An asterisk denotes a significant difference ($p < 0.05$) between baseline and post diet change

3.3 | Fecal bacteria

A three-dimensional ordination solution for NMDS (stress = 0.12) analysis revealed a significant change in fecal bacterial community structure due to the diet change (PERMANOVA, $F = 22.69, p < 0.001$; Figure 5) and individual animal (PERMANOVA, $F = 2.377, p = 0.009$). Furthermore, there was a significant interaction between treatment (diet change) and animal (PERMANOVA, $F = 3.31, p = 0.001$).

4 | DISCUSSION

This study was conducted on four giraffe at CMZ with different sexes, ages, and reproductive states. However, all four giraffe spent more time feeding and less time performing tongue and mouth stereotypies,

TABLE 5 LSM \pm SEM salivary and serum biomarkers before and after an increase in hay-to-grain ratio in four Masai giraffe

Source	Factor	Baseline	Post-diet change	p-value ^a
		LSM \pm SEM	LSM \pm SEM	
Salivary	Insulin (ng/ml)	0.82 \pm 0.24	0.43 \pm 0.05	0.03*
Serum	Insulin (ng/ml)	0.58 \pm 0.09	0.41 \pm 0.08	0.02*
Serum	Glucose (ng/ml)	85.63 \pm 1.55	77.48 \pm 2.05	0.04*
Serum	Insulin/Glucose (mU/mmol)	3.55 \pm 0.44	2.75 \pm 0.46	0.048*
Serum	Leptin (ng/ml)	204.17 \pm 6.96	181.97 \pm 8.32	0.03*
Serum	Ca (mg/dl)	9.62 \pm 0.11	9.43 \pm 0.15	0.14
Serum	P (mg/dl)	5.01 \pm 0.28	5.19 \pm 0.29	0.73
Serum	Ca/P	1.9 \pm 0.11	1.8 \pm 0.12	0.51

Baseline and Post-Diet Change Least Square Means (LSM) were calculated using a GLMM.

^aan asterisk denotes a significant change of $p < 0.05$ between baseline and post diet change.

people-directed behaviors, and alert behaviors following an increase in the hay to grain ratio offered in the diet. There were no observed changes in pacing or licking behaviors. In addition, salivary insulin, and serum insulin-to-glucose ratio decreased, while serum Ca:P was maintained before and after the diet change. Fecal bacterial

community structure also exhibited a highly significant shift post diet change, with differences explained by both treatment effects and individual animal. Taken together, we believe these results show promise and warrant further investigation into the impact of diet on health and welfare in zoo-housed giraffe.

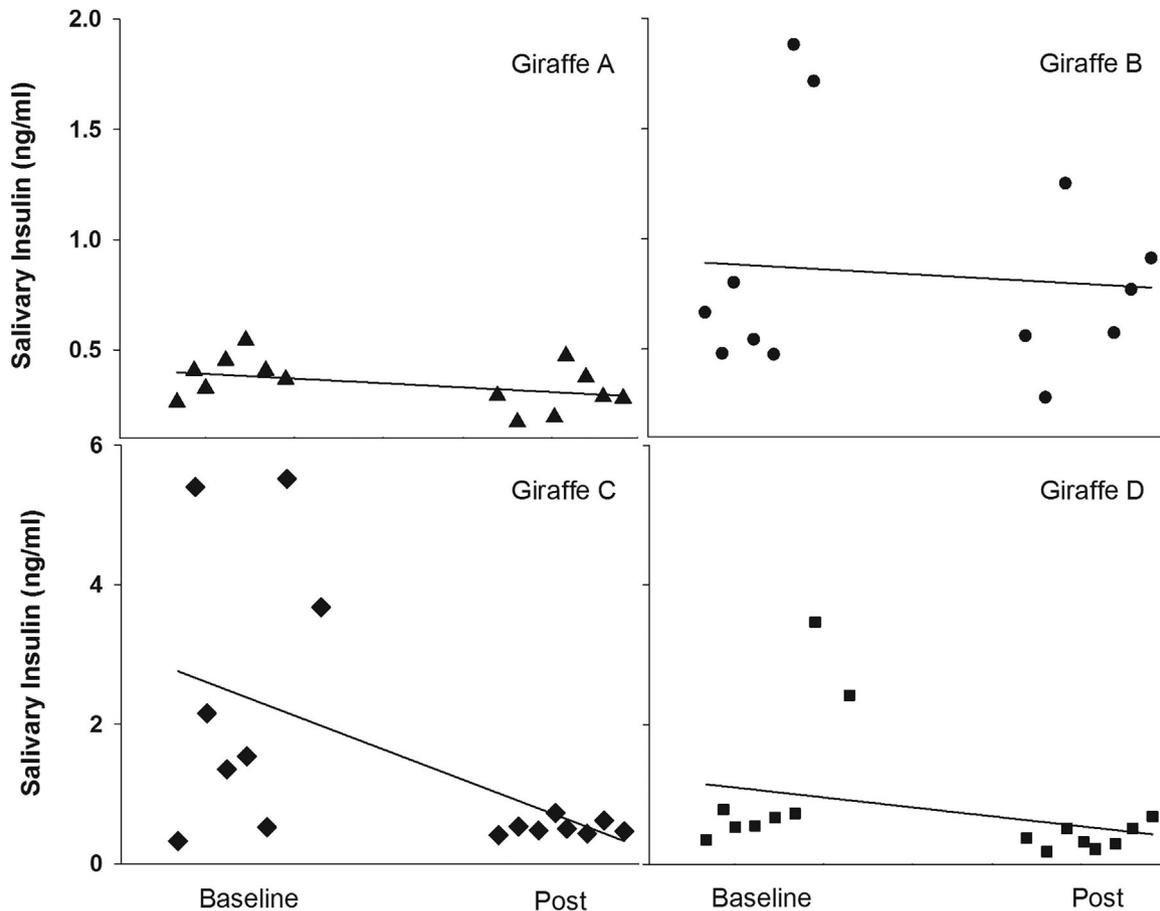


FIGURE 3 Salivary insulin concentrations before (baseline) and after (post) an increase in hay-to-grain ratio in four Masai giraffe. Saliva was collected weekly during both baseline and post periods. Linear regression lines were fit to each individual's set of saliva samples and are represented by solid lines. Note that panels A and B have a different Y axis than panels C and D

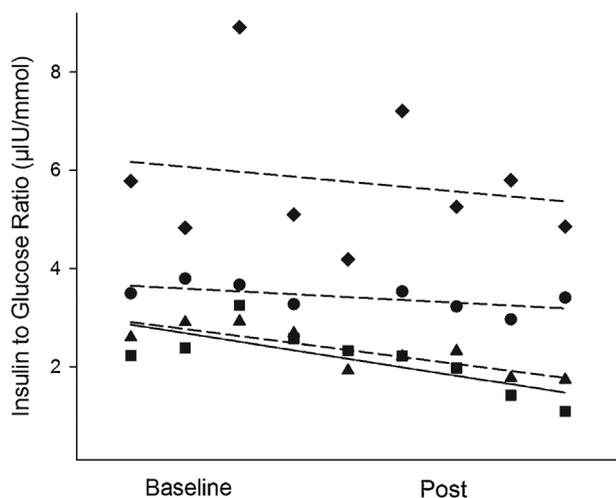


FIGURE 4 Changes over time in serum insulin-to-glucose ratio in four Masai giraffe. Samples taken during the baseline period are to the left of the vertical line while those taken during the 8 weeks post-diet change are to the right of the vertical line. Serum was collected approximately every 2 weeks during both baseline and post periods. Giraffe a, b, c, and d are represented by triangles, circles, diamonds, and squares, respectively (see Table 1). Linear regression lines were fit to each individual's set of serum samples and are represented by the dotted lines

The observed increase from 44% to 51% of time spent feeding over the course of the diet change better approximates the time spent feeding and foraging by wild giraffe that generally range from 50% to 70% (Ginnett & Demment, 1997; Pellew, 1984). This was also greater

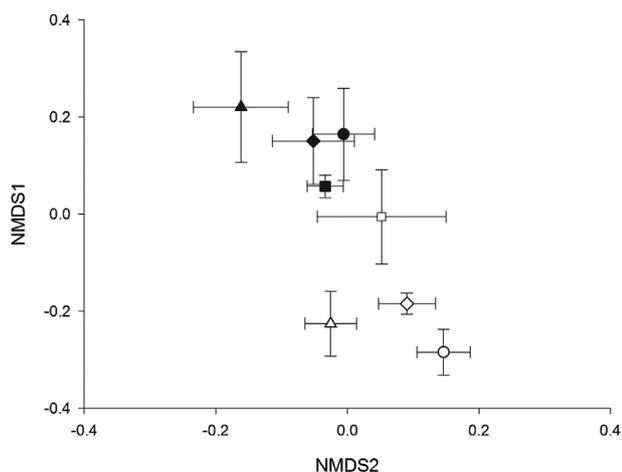


FIGURE 5 NMDS ordination plot of mean axis score \pm SEM individual Masai giraffe fecal bacterial community structures from baseline (open shapes) and post diet change (closed shapes). Individual animals a, b, c, and d are represented by triangles, circles, diamonds, and squares, respectively (see Table 1). PERMANOVA analysis revealed a significant change in community structure due to diet change and individual animal with an interaction between diet change and animal ($p < 0.01$ for all). Points farther apart in space on the plot are less similar in bacterial community structure than points closer together

than the 27.5% of time performing feeding related behaviors observed in a 2006–2007 study at CMZ (Reber, 2008) and 12% time feeding reported in six giraffe consuming a 50:50 ratio of hay to grain at Lincoln Park Zoo (Schmidt & Barbiers, 2005, p. 41). Since completion of the current study, the giraffe at CMZ have been given an ad libitum alfalfa supply to further increase feeding times above 51% of time.

Although increased feeding time was a desired outcome for this study due to potential health benefits, further studies are needed to elucidate any relationship between increased foraging time and direct health outcomes in giraffe. Though not measured in this study, saliva production increases during feeding and ruminating in other species (Bailey & Balch, 1961), which may promote a more balanced rumen pH. In addition, increased feeding time may result in greater phosphorus excretion in feces (rather than urine), as saliva supplies up to 80% of endogenous fecal phosphorus (Bailey & Balch, 1961; Bravo et al., 2003; Horst, 1986). Thus, an increase in the hay-to-concentrate ratio may increase in saliva production and fecal phosphorus excretion, lowering the potential for urolithiasis. Likely a by-product of the increased time spent foraging, giraffe in this study also spent more time stationary within the winter holding space. This was not a desired outcome of the study and future efforts to dividing forage among various feeders may encourage more locomotion between feeding locations.

There was a positive correlation between oral stereotypies and proportion of concentrate fed to giraffe, heifers, and other ungulates (Baxter & Plowman, 2001; Bergeron, Badnell-Waters, Lambton, & Mason, 2008; Redbo & Nordblad, 1997). Correspondingly, tongue and mouth stereotypies decreased by half in the present study following an increase in hay-to-grain ratio, and approached rates (0.8% of time) reported in free-ranging giraffe (Veasey, Waran, & Young, 1996). This is likely linked to the increased time it took to manipulate, consume, and ruminate a larger volume of forage, thereby providing more oral stimulation (Luginbuhl, Pond, Burns, & Russ, 1989). Our results show a lower percentage of tongue/mouth stereotypies than data from a 2006–2007 study at the same zoo in which time spent performing stereotypies varied, but reached as high as 35% of time (Reber, 2008). Despite these improvements, we observed no changes in time spent licking objects, and further research is needed into ways to mitigate this type of stereotypy. Pacing rates did not change during the study, remaining at under 2%. Pacing may be related to time spent confined to the barn during the winter months, as it was only observed at 0.14% of time in a study conducted in the summer of 2014 when giraffe were outside at the same zoo (unpublished observations). While more research into the husbandry of giraffe needs to be done to reach the rates of stereotypies seen in wild conspecifics, this study suggests that offering more forage has the potential to decrease tongue mouth stereotypies in giraffe managed in zoos. Baxter and Plowman (2001) concluded that stereotypic behavior in giraffe can be viewed as a proxy for lower welfare and therefore, the reduction of tongue and mouth stereotypies during this study may be viewed as a positive change.

Increased alert behaviors correlate to increased cortisol levels in other mammals (Carlstead, Brown, & Strawn, 1993) and both alert (also called vigilance) and stereotypic behaviors can increase due to

environmental stressors (Chamove, Hosey, & Schaetzel, 1988). Welp, Rushen, Kramer, Festa-Bianchet, and De Passille, (2004) suggested that cows adjust alert (vigilance) behaviors according to their fearfulness of objects or environments, which may make alert behavior a possible welfare indicator. However, the decrease in people-directed and alert behaviors in the current study may have also been a byproduct of a decreased amount of time spent looking for keepers to deliver food items, since food was more readily available and in increased volumes throughout the day.

The observed decrease in salivary and serum insulin, serum glucose and serum insulin-to-glucose ratios post diet change may be due nutrients being fed in a larger volume of forage, resulting in decreased consumption rate and increased time fermenting and digesting the larger volume of food. Larger particles are retained in the reticulo-rumen for a longer amount of time than small particles with the same density (Kaske & Engelhardt, 1990), altering fermentation rate in the rumen and nutrients available to the abomasum and small intestine between different hay-to-concentrate ratios. Reports of lower insulin and glucose when cows were fed high forage, low concentrate diets compared to those fed high concentrate, low forage diets (Jenny, Polan, & Thye, 1974; Schoonmaker, Loerch, Rossi, & Borger, 2003) support the findings in our study. Based on reports in other species including dairy cows, the observed decrease may also indicate an overall improvement in insulin sensitivity (Guerrero-Romero & Rodriguez-Moran, 2001; Holtenius & Holtenius, 2007; Katz et al., 2000). In addition, this is the first known report of salivary insulin in this species, and the fact that changes in salivary concentrations mirrored trends in serum demonstrates the potential for salivary insulin as a non-invasive metabolic biomarker in this species.

Despite maintaining body weight throughout the study, one giraffe exhibited a gradual and consistent decline in leptin concentrations throughout the study, which may indicate an overall decrease in body fat. This female was in her first trimester of pregnancy and did show a slight, but not significant, increasing trend in body weight throughout the study. Contrary to the pattern observed in the pregnant female in our current study, early pregnancy is associated with progressively increasing serum leptin concentrations in humans and primates (Hardie, Trayhurn, Abramovich, & Fowler, 1997) as well as sheep (Ehrhardt, Slepetic, Bell, & Boisclair, 2001). Thus, future studies are needed to elucidate the significance, if any, of the slight decrease in leptin observed in this individual.

Although we observed increased feeding time, there was no change in the serum phosphorus, calcium or Ca:P. However, the giraffe were above the 1:1 serum Ca:P recommended for ruminant browsers (Miller et al., 2010) prior to the diet change. This ratio is believed to provide amounts of phosphorus needed for metabolic needs without putting the animal at risk to develop rumen acidosis and uroliths (Miller et al., 2010). The giraffe also had a seemingly higher average Ca:P after the diet change (1.8:1) than a small subset of free ranging giraffe from Schmidt et al. (2011) at 1.1:1. The reported dietary phosphorus supplied to our giraffe both before and after the diet change falls within the recommended 0.35% to 0.5% dry matter basis range

recommended by the Giraffe Nutrition Workshop (Schmidt & Barbiers, 2005). However, we used manufacturer reported nutrient content for the diet analysis of both the pellets and ration balancer rather than analysis of what was fed, which is a potential limitation of evaluating nutrient analysis in this study. Phosphorus absorption is directly related to dietary intake while calcium absorption is on an "as needed basis" by the animal in a well-controlled homeostasis (Reinhardt, Horst, & Goff, 1988). Therefore, given the fact that Ca:P were not inverted prior to the diet change, the lack of significant change in the Ca:P is not surprising.

For this study, we explored whether changes in the form of a diet induced changes in fecal bacterial community structure. Our TRFLP method detected highly significant changes in bacterial communities but did not identify specific bacterial family or species differences. Very little work has been done with giraffe microbial communities. AlZahal, Valdes, and McBride (2015) recently reported TRFLP and 454-pyrosequencing analysis completed on single fecal samples from five different giraffe at a different zoo and found high inter-sample similarity attributed to uniformity of their husbandry and feeding management. However, no work has been done looking at the influence of diet on giraffe gut bacteria in this species. de Menezes et al. (2011) found differences in rumen microbial communities via TRFLP in dairy cows fed pasture or a total mixed ration composed of maize silage, concentrate blend, grass silage, molasses, and straw. Many have postulated that different diets result in different rumen metabolites available for lower gastrointestinal activity, thereby altering the lower gastrointestinal bacterial community structure (Ellis et al., 2008; Stewart, Flint, & Bryant, 1997). In future studies, further genetic sequencing of the bacterial DNA will allow a more detailed look at the bacterial community structure changes due to increased hay-to-grain ratio.

There are no reports of insulin/glucose measurements or leptin in either free ranging or zoo-housed giraffe. Thus, this study represents the first information for salivary or serum metabolic indicators for giraffe. Future studies to establish reference ranges for salivary and serum markers of insulin, glucose and leptin in free-ranging giraffe would provide valuable additional information. In addition, comparison of gut microbial communities between zoo-housed and free-ranging individuals would provide groundwork for studying the influence of various diets on health in these specialized browsers. This first investigation included only four giraffe from a single institution, and more studies are needed to establish ideal diets for giraffe in zoos and mitigate all forms of stereotypy. However, we believe the behavioral and physiological changes observed following an increase in hay-to-grain ratio in the current study show promising results that warrant further investigation. The highly significant decreases in time spent displaying people directed and alert behaviors by one of the four giraffe (giraffe B) in the study highlights the importance of monitoring welfare on an individual level. In addition, the reduction in tongue and mouth stereotypic behaviors and increase in time spent feeding in all four giraffe better match levels seen in the wild and underscore the need for more research on the influence of diet on welfare in animals managed under human care.

5 | CONCLUSIONS

1. Increased hay-to-grain ratio in the Masai giraffe diet at CMZ increased time spent feeding and decreased some stereotypic behaviors, salivary, and serum insulin, and insulin to glucose ratios, warranting further investigation into dietary impacts on measures of health and welfare in giraffe.
2. Fecal bacterial communities changed dramatically with alterations in the form of diet; further research is needed to elucidate the relationships between this and rumen health.
3. Validation of new measures of health will allow future research into metabolic markers and fecal bacteria in wild and zoo-housed giraffe and will help guide future developments in improving zoo-housed ruminant diets.

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CONFLICTS OF INTEREST

The authors have received no financial benefits from this publication and have no conflicts of interest to report.

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