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Research article

In-vitro fermentation of commonly fed forage using African elephant *Loxodonta africana* faecal inoculum

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Abstract

Gastrointestinal issues and elevated body condition scores are concerns for human-managed African elephants *Loxodonta africana*. Thus, research to formulate appropriate feeding programmes is paramount. Fermentability of seven commonly fed types of forage were studied in-vitro using faeces from human-managed African elephants as an inoculum source. Air-dried plant samples (0.5 g) from various harvest seasons [timothy hay (n=4 seasons), N&S grass (n=2), alfalfa hay (n=3), tulip poplar (n=2), thorny elaeagnus (n=3), sweet gum (n=3) and willow oak (n=3)] were incubated with buffered faecal inoculum (n=4 elephants). Gas production was measured over 72 hr and concentration of short-chain fatty acids (SCFA) and ammonia at 72 hr. The fermentation parameters varied widely among plant species (P<0.001) with grass and legume species being more fermentable than browse species. Gas production ranged from 22 ml/g organic matter (OM) for willow oak to 140 ml/g OM for alfalfa hay and SCFA from 1.38 (willow oak) to 5.43 (alfalfa hay) mmol/g OM. Within forage, differences in fermentability (P<0.05) were found between harvest seasons for timothy hay, N&S grass and alfalfa hay (for total SCFA 7 to 23% deviation from the average) but this effect was limited or absent for the browse species. Total SCFA correlated with dietary fibre (R^2=0.477, P<0.001), lignin (R^2=0.432, P=0.002) and with non-starch polysaccharide + lignin (R^2=0.637, P<0.001). It is recommended to consider fermentative capacity of evaluated forage species and also harvest season for the grass and legume species in African elephant feeding management programmes to assure elephant body condition and nutritional health.

Introduction

The health and welfare of African elephants *Loxodonta africana* in zoological institutions is promoted by feeding strategies that meet their behavioural and nutritional requirements. Common nutrition-related health and welfare problems include being overweight and obesity (Morfeld et al. 2014; Edwards et al. 2019), which are caused by an energy intake that exceeds expenditure and is associated with foot problems, reproductive difficulties and insulin resistance (Greco et al. 2016; Morfeld and Brown 2016). Overconsumption of highly digestible forage and lack of activity may underlie obesity in elephants managed in human care (Dierenfeld 2006). The forage commonly used to feed elephants in human care may contrast with their natural foraging ecology. Free-ranging African elephants forage for 48–63% of daylight hours with approximately 56% of the time spent manipulating browse and 45% grazing (Dougall and Sheldrick 1964; Tchamba and Seme 1993; Chiaki 1996). However, time spent feeding does not directly correlate to intake of mass, as browsing requires more manipulation and processing prior to ingestion than does grazing. Furthermore, they also have profound seasonal changes and habitat-attributed differences in browse and grass consumption with higher browse consumption in the dry season and higher grass consumption in long-grass regions as compared to thicket areas (Laws 1970; Barnes 1982). When available, grasses, creepers and herbs are the predominant consumed forage and are preferred over browse. The consumption of browse in the dry season is related to the decreased availability of grasses, creepers and herbs in this season (Bax and Sheldrick 1963). Natural diets are

typically high in fibre with crude fibre levels ranging from 21 to 49% of dry matter (DM) (Dougall and Sheldrick 1964). Elephants have a relatively rapid gut transit time (38–48 hours for alfalfa hay; Foose 1982) meaning their digestive system needs considerable amounts of indigestible feed to maintain digestive tract health (Benedict 1936; Bax and Sheldrick 1963; Rees 1982; Hackenberger 1987).

Since free-ranging African elephants mainly consume browse and grass, zoological institutions have searched for local alternatives. A list of over 80 plant species fed to African elephants in zoos in North America includes a variety of trees, grasses, herbs and legumes (Olson 2002). The cell walls of plant species differ in composition with varying forms and amounts of hemicellulose (12–29% of DM), cellulose (13–34% of DM), and lignin (6–19% of DM) (Hummel et al. 2006a). Furthermore, plant crude protein (CP) contents have been shown to vary between species from 7% DM for guinea grass *Megathyrsus maximus* to 25% for white leadtree *Leucaena leucocephala* (Singh et al. 2014). These variations in chemical composition also suggest that nutritional value varies among forage. Moreover, chemical compositions of forage may differ among season of harvest and stage of maturity. As seasons progress from spring to winter, leaves mature, flowers and seeds develop, lignification takes place, and deciduous species drop their leaves and translocate resources to the roots (Raven et al. 2012a, b). Maturation of 1 year old pure ryegrass *Lolium perenne* for 90 days resulted in profound changes in CP (24% to 7% DM) and neutral detergent fibre (NDF; 43% to 65% DM) (Chaves et al. 2006). Scogings et al. (2004) found a CP increase of 8–56% between the winter and summer stages of two out of three browse species. Similarly, all plants in this prior study showed a decline of 15 to 77% in either cellulose or lignin content when transitioning from summer to winter. Besides these factors contributing to variation in nutrient composition between and within forage and their specific parts, other factors such soil conditions, handling and storage further increase variation. Studies characterising (parts of) foragers are therefore warranted to understand how these can be incorporated in feeding programmes in zoos.

The differences in chemical composition between fibrous forage and seasonal variations in composition translate into varying degrees of fermentation by gastrointestinal microbes. In-vitro fermentation using microbes from the gastrointestinal tract is commonly used to evaluate and compare nutritional properties of forage (Coles et al. 2005). In-vitro incubation of sheep rumen microbiota with browse twigs for 72 hr resulted in 127 ml/g DM gas, whereas incubation with grasses led to twice as much gas (255 ml/g DM) (Hummel et al. 2006a) and incubation with summer-harvested Acacia leaves *Acacia saligna* for 24 hr resulted in 133 ml/g DM, whereas winter harvests yielded 158 ml/g DM (Salem 2005). The amount of lignin in forage is an important characteristic and is associated with lowering the degradability of forage (Smith et al. 1972; Goto et al. 1991; Hummel et al. 2006a; He et al. 2018). Such relations are valuable as one might (crudely) estimate the potential microbial degradability based on key chemical components.

Most studies evaluating the in-vitro fermentability of browse and grasses used as forage in zoos are based on microbiota from the rumen (Salem 2005; Hummel et al. 2006), which might have different fermentative capacities than the large intestinal microbiota from elephants. To gain insight in variations in fermentability of forage fed to African elephants in human care and their potential contributions to overall energy intake, this study evaluated the in-vitro fermentation of seven commonly-fed types of forage using faecal inocola from elephants. The effect of season of harvest was evaluated within forage and the relation between chemical composition and in-vitro fermentability was explored.

Materials and Methods

Fermentation substrates

Forage evaluated for fermentability were part of a larger study described in Wood et al. (2020). A total of 20 samples from seven types of forage were fed during different North Carolina seasons from February 2016 to April 2017. The five browse/tree species were collected every 6 weeks and grouped into the appropriate season. They were collected from the same designated browse collection areas of the NC Zoo in Asheboro (North Carolina, USA). The two hay species were collected from the shipments purchased from the same commercial provider numerous times throughout the year (Table 1). Seasons were spring (March–May), summer (June–August), autumn (September–November), and winter (December–February). Portions of the hay that was fed during each season likely represent those grown during the prior season. Thus, these data represent forage as fed at the NC Zoo but not necessarily as grown. Not all plant species were harvested during every season due to collection limitations and varying seasonal availability. The plant species were timothy grass *Phleum pratense*, thorny elaeagnus *Elaeagnus pungens*, willow oak *Quercus phellos*, sweet gum *Liquidambar styraciflua*, alfalfa *Medicago sativa*, tulip poplar *Liriodendron tulipifera* and elephant habitat grasses from the neighbouring North and South enclosures (N&S grass). N&S grass consisted of tall fescue *Festuca arundinacea,* annual ryegrass *Lolium multiflorum*, bermuda grass *Cynodon dactylon*, wild white clover *Trifolium repens* and limited weed species planted or found consistently on both enclosures. The exact ratios of these grass, legume and weed species were not determined in this study due to the limited time researchers were given access to the elephant pastures. However, these listed species offer a representation of what the elephants were grazing. For the browse samples, it was observed that all parts offered were consumed by elephants, therefore stems, leaves, bark and branches (≤5 cm) were included in the samples. All attempts were made to mimic the seasonal browse parts eaten by elephants within the representative samples for analyses (i.e. fewer leaves in winter than in summer). Repeated samples were collected throughout the season's months (once every 6 weeks) and pooled to represent one seasonal sample for a specific plant species. Fresh samples were collected, stored and processed as previously described in Wood et al. (2020). Powdered soluble starch (S9765, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA) and air-dried ground perennial ryegrass silage *Lolium perenne* were used as control substrates. Powdered soluble starch contained 80.5% DM and on DM basis 99.7% organic matter (OM), and 0% CP. Air-dried standard grass contained 92.3% DM, and on DM basis 89.8% OM, and 16.3% CP.

Faecal donors and faeces collection

Four female African elephants from Safaripark Beekse Bergen (Hilvarenbeek, the Netherlands) were used. They differed in age (13, 26, 32 and 33 years), body weight (respectively 2015, 3020, 3345 and 3540 kg), and shoulder height (respectively 2.17, 2.47, 2.55 and 2.60). The 26-year old elephant and her 13-year old daughter were housed in a family group of four elephants. The 26-year old elephant was lactating. The remaining two elephants were housed together as a separate group. The family group had an outside enclosure of 13,000 m^2 and an inside enclosure of 450 m^2 that included three compartments (each ~16 to 25 m^2), whereas the other group had an outside enclosure of 3,500 m^2 and two inside compartments of 42 m^2 each. No health problems were registered for any of the elephants. All elephants had unlimited access to water and were fed according to maintenance with 30–40% of their daily allowance in the morning and 60–70% in the evening. The diet fed for at least 6 months consisted mainly of

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Table 1. Chemical composition* of seven types of forage harvested in different seasons** and commonly fed to African elephants *Loxodonta africana* in the USA[1].

*DM=dry matter, OM=organic matter, Cfat=crude fat, CP=crude protein, NDICP=neutral detergent insoluble crude protein, ADICP=acid detergent insoluble crude protein, NFC=non-fibre carbohydrates, WSC=water soluble carbohydrates, ESC=ethanol soluble carbohydrates, aNDFom=amylase and sodium sulphite treated neutral detergent fibre corrected for residual ash, ADF=acid detergent fibre, ADL=acid detergent lignin, DF=dietary fibre, NSP=non-starch polysaccharides; DM expressed on % as is basis and other parameters on % of DM. **All four seasons were not available for all seven types of forage. Seasons are defined as: spring (March–May), summer (June–August), autumn (September–November) and winter (December–February). [1]More diet and forage information from NC Zoo African elephants can be found in Wood et al. (2020). [2]Timothy grass *Phleum pratense*, alfalfa *Medicago sativa*, tulip poplar *Liriodendron tulipifera*, thorny elaeagnus *Elaeagnus pungens*, sweet gum *Liquidambar styraciflua*, and willow oak *Quercus phellos*. N&S grass consisted of tall fescue *Festuca arundinacea*, annual ryegrass *Lolium multiflorum*, bermuda grass *Cynodon dactylon*, wild white clover *Trifolium repens* and limited weed species. The exact ratios of these grass, legume and weed species were unknown.

Forage[2]	Season	DM	OM	Cfat	CP	NDICP	ADICP	NFC	WSC	ESC	Starch	aNDFom	ADF	ADL	DF	NSP
Timothy hay	Spring	91.0	93.1	2.6	7.4	2.0	0.9	20.4	14.2	4.9	0.9	62.8	40.4	7.3	77.3	70.0
	Summer	45.3	91.3	3.2	10.7	3.9	1.2	14.3	8.0	3.7	1.3	63.3	35.3	3.5	72.6	69.2
	Autumn	89.6	94.2	2.3	8.7	2.4	0.8	20.8	16.0	7.5	0.8	62.4	35.0	4.3	75.0	70.7
	Winter	87.9	93.5	2.6	10.1	2.9	0.8	20.3	17.0	8.6	0.8	60.7	31.7	3.2	71.4	68.3
N&S grass	Summer	25.2	85.7	3.1	18.3	7.7	1.4	8.2	6.3	5.0	2.3	51.4	31.9	5.7	57.1	51.5
	Winter	39.0	82.7	2.0	17.4	5.7	1.8	14.3	9.7	5.0	2.4	50.1	40.2	8.4	55.8	47.5
Alfalfa hay	Spring	88.4	90.8	2.2	20.4	3.8	1.9	22.9	7.4	5.5	0.5	45.3	37.9	9.2	62.2	53.1
	Summer	86.8	91.9	1.8	11.9	2.7	1.8	14.1	4.4	2.1	0.5	64.0	52.0	12.8	75.6	62.9
	Winter	91.6	88.7	2.4	22.3	4.2	1.5	22.9	8.1	4.6	1.4	41.2	35.8	8.7	58.0	49.4
Tulip poplar	Summer	43.5	95.3	5.4	6.1	3.9	3.0	31.8	6.7	6.0	4.8	52.2	39.8	13.6	73.2	59.7
	Autumn	42.5	94.8	4.4	7.5	4.2	2.8	21.7	7.5	5.4	3.7	61.4	40.6	10.8	73.9	63.2
Thorny eleagnus	Spring	55.9	96.7	1.7	11.7	4.1	2.7	12.4	5.1	3.4	2.7	70.9	58.2	21.6	77.3	55.7
	Autumn	44.5	95.8	2.7	13.5	5.3	3.0	7.9	2.8	2.6	0.4	71.8	56.5	22.2	76.6	54.4
	Winter	48.0	95.8	2.2	15.7	5.2	3.2	11.1	6.7	4.1	1.5	66.7	55.9	21.9	72.2	50.2
Sweet gum	Summer	42.2	95.2	2.3	6.6	5.1	4.0	37.3	7.2	5.5	2.2	49.1	39.7	11.2	78.7	67.5
	Autumn	44.1	94.2	1.9	6.4	4.6	4.3	35.0	13.0	7.3	3.0	51.1	36.8	10.5	75.8	65.3
	Winter	50.1	95.5	1.5	4.8	3.1	2.2	21.9	7.5	5.0	2.3	67.4	56.3	15.6	82.0	66.4
Willow oak	Spring	47.6	95.7	2.9	8.6	4.6	2.8	25.7	5.7	5.6	0.4	58.6	42.5	13.7	78.3	64.7
	Summer	56.6	95.6	1.9	7.2	4.9	3.2	23.8	8.4	6.2	2.0	61.6	53.7	22.0	78.4	56.4
	Autumn	60.1	95.9	1.7	6.8	4.1	3.0	22.7	10.7	6.8	3.0	64.9	47.7	13.6	77.8	64.2

meadow grass hay (mixed species) supplemented with fruits and vegetables. Cakes (Salvastar PS mit Äpfeln und Karotten, Salvana Tiernahrung GmbH, Klein Offenseth-Sparrieshoop, Germany) were provided to meet the mineral and vitamin requirements. The lactating female had access to extra alfalfa hay to prevent weight loss.

Elephants were individually housed in their boxes or compartments for faeces collection, except for the lactating female who was housed with her calf. Immediately after defecation the elephants were released from the compartment by the caretakers after which the faeces were collected. For each elephant, one large shovel scoop of manure was collected, of which a grab sample, consisting of 8–12 spots, was prepared. The grab samples were divided between two CO_2 pre-flushed containers and afterwards flushed with a bottle of CO_2 to maintain anaerobic conditions. Materials used for collection were sterilised using 70% ethanol to prevent contamination. Time between defecation and end of faecal collection did not exceed 11 min. All faeces were collected within 1 hr and transported within 2 hr to the Animal Nutrition Group laboratory (Wageningen University & Research, Wageningen, the Netherlands).

In-vitro fermentation and sampling

Per elephant, one faecal inoculum was prepared by combining the faecal sample with an anaerobic and pre-warmed (36.5°C) buffer solution under continuous flushing with a CO_2 buffer (Cone et al. 1996) in a ratio of 1:3 (mass/volume) (Desrousseaux et al. 2012). After mixing for 60 sec using a hand-blender the mixture was strained through a double layer of cheesecloth. The resulting inoculum was dispensed in portions of 60 ml into pre-warmed 300-ml fermentation bottles, pre-flushed with CO_2, and contained 0.45–0.55 g of substrate or no substrate (blanks). Bottles were attached to an automated gas production system (Cone et al. 1996) and incubated, with one replicate per substrate-inoculum combination, in shaking water baths for 72 hr at 36.5°C (i.e. body temperature of African elephants; Mole et al. 2016).

Chemical analyses

Forage samples were analysed for composition as described in Wood et al. (2020) using Dairy One Forage Labs. Water soluble carbohydrates (WSC) and ethanol soluble carbohydrates (ESC) were analysed using Hall et al. (1999) methodology for neutral detergent soluble carbohydrates partitioning. For WSC (simple

Table 2. Fermentation parameters* at 72 hr incubation for seven types of forage by harvest season commonly fed to African elephants *Loxodonta africana* in zoological institutions in the USA. *OMCV=organic matter corrected volume, SCFA=short chain fatty acids. OMCV expressed on ml/g OM basis, SCFA on mmol/g OM and NH_3 in mg/g OM. **SEM=standard error of the mean. ***P-values for main effects of the model and values with different superscripts indicate significant difference between types of forage (P<0.05).

Parameter	Forage[1]							SEM**	P-value***	
	Timothy hay	N&S grass	Alfalfa hay	Tulip poplar	Thorny elaeagnus	Sweet gum	Willow oak		Forage	Season
OMCV	132^{a}	128^{a}	140^{a}	84^{b}	39^{c}	33^{c}	22^{c}	10.2	<0.001	0.182
Total SCFA	4.48^{a}	5.09^{a,b}	5.43^{b}	3.32^{c}	2.33^{d}	1.48^{e}	1.38^{e}	0.36	<0.001	0.065
Acetic acid	2.67^{a}	3.30^{b}	3.59^{b}	2.35^{a}	1.52^{c}	1.07^{c,d}	0.98^{d}	0.22	<0.001	0.107
Propionic acid	1.13^{a}	1.07^{a}	1.16^{a}	0.73^{b}	0.49^{b,c}	0.27^{c,d}	0.23^{d}	0.15	<0.001	0.415
Butyric acid	0.53^{a}	0.54^{a}	0.45^{a,b}	0.18b,^{c}	0.19^{c}	0.09^{c}	0.12^{c}	0.08	<0.001	0.572
Isobutyric acid	0.03^{a}	0.05^{b}	0.06^{b}	0.01^{c}	0.02^{a}	0.01^{c}	0.01^{c}	0.01	<0.001	0.089
Isovaleric acid	0.04^{a}	0.07^{b}	0.08^{b}	0.01^{c}	0.03^{a}	0.01c	0.01^{c}	0.01	<0.001	0.052
Valeric acid	0.08^{a,b}	0.06^{a,c}	0.09^{a}	0.03^{b,c}	0.06^{a,c}	0.02^{c}	0.03^{c}	0.02	<0.001	0.242
NH_3	29.5^{a}	44.2^{d}	44.7^{d}	24.2^{c}	36.1^{b}	25.4^{c}	27.3^{a,c}	2.2	<0.001	0.116

sugars and fructan), samples were incubated with water in a 40°C bath for 1 hr then analysed using a Thermo Scientific Genesys 10S Vis Spectrophotometer. For ESC (simple sugars), samples were shaken with 80% ethanol for 4 hr at 180 epm before analysis with a Thermo Scientific Genesys 10S Vis Spectrophotometer. ADF was analysed using ANKOM Technology Method 5 using filter bags with 0.5 g of sample in 2 L of ADF solution in the ANKOM a200 Digestion Unit. Samples were analysed for aNDFom using ANKOM Technology Method 6 and solutions described in Van Soest, et al. (1991) with an ashing step before placing samples in the filter bags. Starch was analysed using a YSI 2700 SELECT Biochemistry Analyzer after a 40°C water bath incubation and filtration on Whatman 41 filter paper. ADICP, or the protein bound to ADF, was analysed from the ADF residue using a Leco TruMac N Macro Determinator while NDCIP was determined from aNDF without the use of sodium sulphite and analysed using the same Determinator. The non-fibre carbohydrate (NFC) fraction was calculated from the following equation 100% − (CP% + (NDF% - NDICP%) + Fat% + Ash%), dietary fibre (DF) fraction as 100% − (CP% + Fat% + Ash% + Starch% + ESC%) and NSP as DF% - ADL%. Samples of the inocula (t=0) and fermentation fluids at end of incubation (t=72 hr) were taken. For SCFA analysis, samples were mixed 1:1 with phosphoric acid (H_3PO_4) solution and isocaproic acid as internal standard and for ammonia (NH_3) analyses samples were mixed 1:1 with 10% trichloroacetic acid. After centrifugation (5 min, 20,817 ×g, 4°C), SCFA concentrations in inocula and fermentation fluids were analysed by injecting 0.5 µl in a gas chromatograph (Trace 1300, Thermo Fisher Scientific, Waltham, MA, USA) with a split/splitless injector operated in split mode (split ratio 1:36), at a temperature of 260°C, using a capillary column (HP-FFAP, Agilent, Santa Clara, CA, USA; 30 m × 0.32 mm × 0.25 µm) with hydrogen as carrier gas (2.5 ml/min), and fitted to an flame ionisation detector. SCFA were identified and quantified using a chemical standard solution (0.85% M ortho-phosphoric acid) with isocaproic acid (19.681 mM) as an internal standard for correction. After centrifugation (5 min, 20,817 ×g, 4°C), NH_3 concentrations were quantified using a spectrophotometer as described by Bosch et al. (2008).

Calculations and statistical analyses

Gas production in ml was expressed per g organic matter (OM corrected volume; OMCV) as well as SCFA (mmol/g OM) and NH_3 (mg/g OM). Gas production data were checked for recorder malfunctions and, if needed, data for that position were omitted (7 out of 92 incubations). Also, for one incubation the SCFA and NH_3 were too low to be correct and these values were excluded from further analyses. Data for the control substrates and blanks were reported separately and not included in the statistical

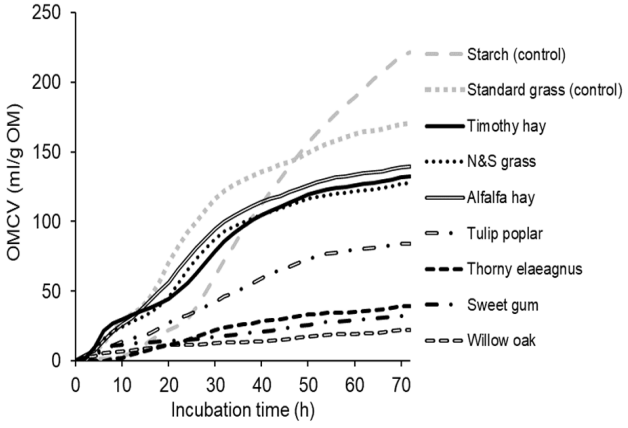

Figure 1. In-vitro gas production (OMCV ml/g OM) over incubation time (hr) of two control substrates and seven types of forage commonly fed to African elephants *Loxodonta africana* in zoological institutions in the USA.

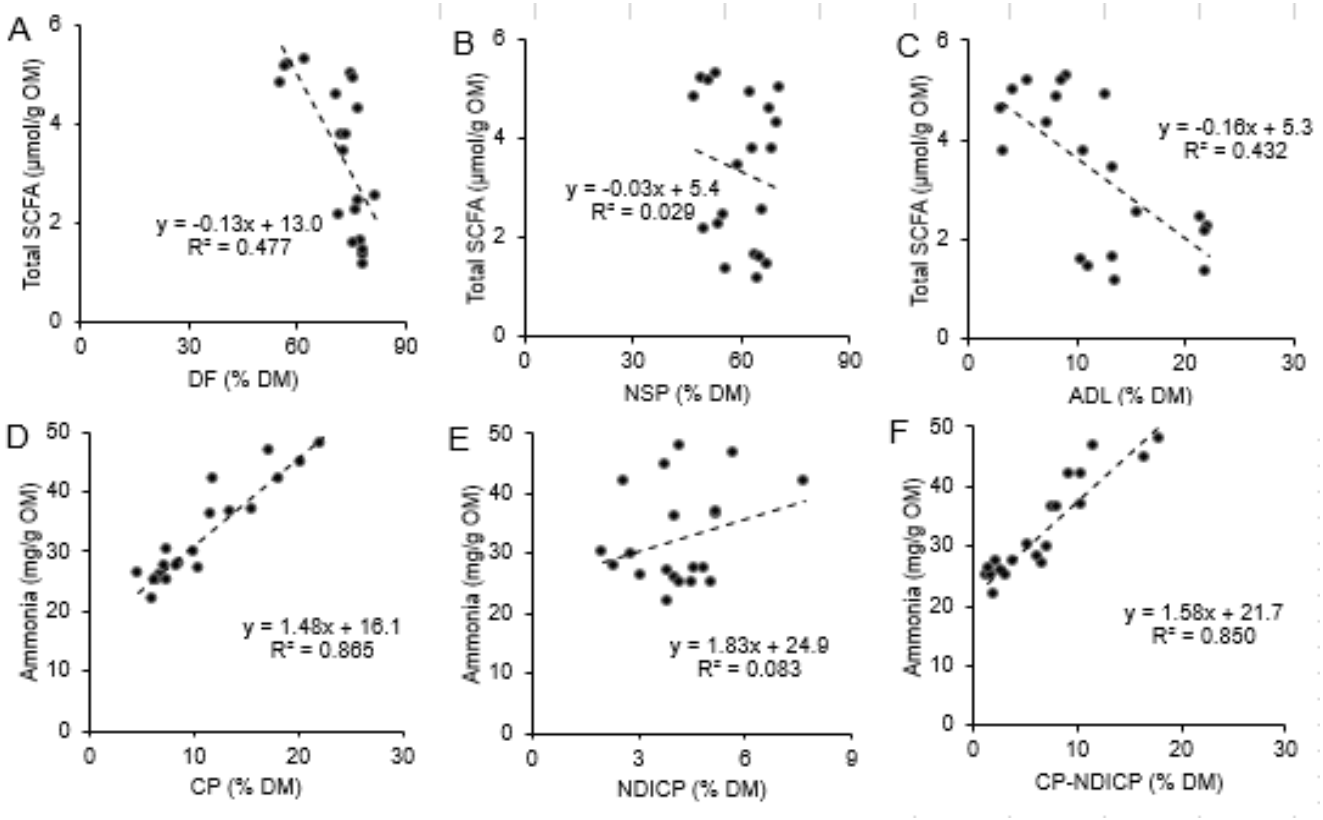

Figure 2. Correlation between the chemical composition and fermentation parameters of seven types of forage commonly fed to African elephants in a zoological institution in the USA. Correlation between dietary fibre (DF; panel A), non-starch polysaccharide content (NSP; panel B) or acid detergent lignin (ADL; panel C) with total SCFA produced at 72 hr and correlation between crude protein (CP; D), neutral detergent insoluble crude protein (NDICP; E) or CP corrected for NDICP (F) with NH_3 produced at 72 hr.

analyses. The SCFA concentrations in fermentation fluids collected after 72 hr of incubation originate from the SCFA in the inoculum (t=0) and those produced during incubation. To estimate the SCFA production from each substrate as a proxy of fermentability, SCFA concentrations at 72 hr were corrected for the SCFA in the inoculum within each faecal donor. Two statistical models were used to evaluate differences between types of forage and between harvest seasons within forage. Differences between types of forage in fermentation parameters were tested using ANOVA by PROC MIXED in SAS 9.3 (SAS, SAS Institute, Cary, North Carolina, USA) with Forage and Season as fixed effects and Elephant as a random effect. In case Forage was significant ($P \leq 0.05$), Tukey-adjusted P-values were used to evaluate significance of differences between types of forage. For the second model, the same response variables were tested within each forage using ANOVA by PROC MIXED with Season as fixed effect and Elephant as a random effect. Least square means (LSM) were calculated and presented for both models. Linear regression was used to test correlations between selected forage chemical components and total SCFA or NH_3 produced at 72 hr of incubation. For total SCFA, the regression model with ADL and NSP was also tested. Differences were considered significant at $P<0.05$.

Results

Fermentation kinetics

The 72-hr gas production varied between the substrates (Figure 1). During the first 10 hr, gas production was more rapid for timothy hay, alfalfa hay, N&S grass and standard grass (control). These substrates also showed higher OMCV values at 72 hr. Starch (control) had low OMCV values for the first 10 hr and then gas production rapidly increased resulting in highest OMCV values at 72 hr. Tulip poplar had an intermediate fermentation rate and OMCV values throughout incubation and thorny elaeagnus, sweet gum and willow oak lowest values.

Differences between types of forage

All samples were high in carbohydrates and relatively low in other nutrients and varied in particular in non-fibre carbohydrates (NFC), water soluble carbohydrates (WSC), neutral detergent fibre on an OM (ash-free) basis (aNDFom), and acid-detergent lignin (ADL) (Table 1) (Wood et al. 2020). Forage differed in terms of fermentative properties ($P<0.001$ for all parameters; Table 2). In line with the gas production kinetics, lowest OMCV at 72 hr were found for thorny elaeagnus, willow oak and sweet gum,

Table 3. Differences in fermentation parameters* at 72 hr of incubation among harvest seasons** within seven types of forage commonly fed to African elephants *Loxodonta africana* in zoological institutions in the USA and control substrates. *OMCV=organic matter correct volume, SCFA=short chain fatty acids, AA=acetic acid, PA=propionic acid, BA=butyric acid, iBA=isobutyric acid, iVA=isovaleric acid, VA=valeric acid; OMCV expressed on ml/g OM basis, SCFA on mmol/g OM and NH_3 in mg/g OM; differing superscripts (a, b) means significant difference at (P<0.05). **Seasons are defined as: spring (March–May), summer (June–August), autumn (September–November) and winter (December–February). ***SEM=standard error of the mean.

Forage[1]	Season	OMCV	Total SCFA	AA	PA	BA	iBA	iVA	VA	NH_3
Timothy hay	Spring	126[a]	4.29[a,b]	2.63[a,b]	1.12	0.41	0.03[a]	0.04	0.08	30.2
	Summer	87[b]	3.42[a]	2.09[a]	0.88	0.38	0.03[a]	0.03	0.04	31.1
	Autumn	146[a,c]	5.02[b]	2.88[b]	1.18	0.78	0.04[b]	0.04	0.11	27.9
	Winter	149[c]	4.87[b]	2.95[b]	1.24	0.53	0.03[a,b]	0.04	0.08	29.6
	SEM***	16	0.56	0.33	0.25	0.20	0.01	0.01	0.04	3.0
N&S grass	Summer	147[a]	5.37[a]	3.54[a]	1.10	0.56	0.05	0.07	0.05	41.9[a]
	Winter	107[b]	4.66[b]	2.95[b]	1.01	0.51	0.05	0.07	0.08	46.7[b]
	SEM	12	0.37	0.26	0.21	0.12	0.01	0.02	0.03	2.2
Alfalfa hay	Spring	134[a]	5.56[a]	3.71[a]	1.18[a,b]	0.44	0.06[a]	0.08[a]	0.08	44.8
	Summer	117[a]	4.50[b]	2.95[b]	0.99[a]	0.39	0.05[b]	0.06[b]	0.07	42.0
	Winter	166[b]	6.11[a]	4.05[a]	1.30[b]	0.47	0.07[a]	0.10[a]	0.13	47.8
	SEM	9	0.34	0.27	0.16	0.11	0.01	0.02	0.03	3.9
Tulip poplar	Summer	83	3.19	2.19	0.73	0.22	0.01	0.01	0.04	22.0
	Autumn	84	3.29	2.41	0.68	0.16	0.02	0.02	0.02	24.9
	SEM	6	0.29	0.13	0.15	0.06	0.01	0.01	0.02	2.1
Thorny elaegnus	Spring	46	2.70	1.74	0.62	0.21	0.03	0.04	0.07[a]	36.2
	Autumn	35	2.10	1.42	0.42	0.17	0.03	0.04	0.04[b]	36.5
	Winter	45	2.45	1.60	0.49	0.22	0.03	0.03	0.08[a]	36.8
	SEM	10	0.28	0.15	0.12	0.05	0.01	0.01	0.02	2.3
Sweet gum	Summer	31	1.45	1.07	0.22[a]	0.12	0.01	0.01	0.02	25.0
	Autumn	32	1.57	1.11	0.34[b]	0.09	0.01	0.01	0.02	25.0
	Winter	30	1.41	1.01	0.24[a]	0.12	0.01	0.01	0.03	26.0
	SEM	10	0.33	0.19	0.09	0.05	0.01	0.01	0.01	1.3
Willow oak	Spring	18	1.11	0.83	0.16	0.08	0.01	0.01	0.02	27.3
	Summer	18	1.26	0.86	0.23	0.12	0.01	0.01	0.03	27.3
	Autumn	24	1.64	1.17	0.27	0.16	0.01	0.01	0.02	25.9
	SEM	9	0.37	0.22	0.09	0.06	0.00	0.01	0.02	1.5
Control grass	n.a.	170	7.42	4.97	1.46	0.77	0.07	0.07	0.08	36.8
Control starch	n.a.	221	6.66	4.61	1.17	0.73	0.04	0.04	0.06	22.3

intermediate value for tulip poplar and highest values for timothy hay, alfalfa hay and N&S grass (P<0.05). Total SCFA produced showed a generally similar pattern with considerable variation among forages. Alfalfa hay and N&S grass had the highest values (5.43 and 5.09 mmol/g OM) whereas willow oak and sweet gum yielded the lowest values (1.38 and 1.48 mmol/g OM). The least acetic acid was produced from thorny elaeagnus, willow oak and sweet gum (0.98–1.52 mmol/g OM), and the most from alfalfa hay and N&S grass (3.30 and 3.59 mmol/g OM). Propionic acid production was the lowest for willow oak and sweet gum (0.23 and 0.27 mmol/g OM), the highest for timothy hay, alfalfa hay and N&S grass (1.07–1.16 mmol/g OM). The least butyric acid production was for thorny elaeagnus, willow oak, sweet gum and tulip poplar (0.09–0.19 mmol/g OM), and the most for timothy hay, alfalfa hay and N&S grass (0.45–0.54 mmol/g OM). Isobutyric, isovaleric and valeric acid production was low, however some variation between samples was found. NH_3 production was the lowest for sweet gum (25.4 mg/g OM) and tulip poplar (24.2 mg/g OM), and the highest was for alfalfa hay (44.7 mg/g OM) and N&S grass (44.2 mg/g OM).

Differences between seasons within forage

For willow oak and tulip poplar, harvest season had no effect on the fermentation parameters (P>0.05; Table 3). Fermentability varied most among harvest seasons for timothy hay, N&S grass and alfalfa hay. For timothy hay, generally higher fermentability, i.e. OMCV and SCFA production, were found for samples from winter and autumn than from summer and spring samples being intermediate. Samples from summer alfalfa hay were generally less fermentable than those from spring and winter. N&S grass had lower OMCV, total SCFA and acetic acid values for winter samples compared to summer samples. Fermentation of N&S grass from winter also had higher NH_3 production than that from

Journal of Zoo and Aquarium Research 8(4) 2020
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summer. For sweet gum, autumn samples had lowest propionic acid production. Autumn samples of thorny elaeagnus yielded lowest valeric acid values. Incubation with starch and standard grass (controls) resulted in high values for OMCV (170 and 221 ml/g OM, respectively) and total SCFA (6.66 and 7.42 mmol/g OM). The NH_3 production was lower for starch (22.3 mg/g OM) than for the standard grass (36.8 mg/g OM). The average total SCFA concentration for the blanks was 15.9 mmol/l, which was close to that for the inocula (14.9 mmol/l).

Correlation between the chemical composition and fermentation parameters

Total SCFA produced at 72 hr correlated with dietary fibre (R^2=0.477, P<0.001; Figure 2), ADL (R^2=0.432, P=0.002) and ADF (R^2=0.277, P=0.017; not shown), but not with NSP (R^2=0.029, P=0.475) and aNDFom (R^2=0.128, P=0.122; not shown). Correlation coefficient increased when NSP and ADL were included in the model (R^2=0.637, P<0.001; total SCFA=11.7–0.10×NSP–0.20×ADL). NH_3 produced at 72 hr correlated with total CP (R^2=0.864, P<0.001) but not with the neutral detergent insoluble CP (NDICP; R^2=0.080, P=0.226). Furthermore, total CP content minus NDICP content also correlated with NH_3 produced at 72 hr (R^2=0.850, P<0.001).

Discussion

This study showed variations in in-vitro fermentability of seven types of forage fed to African elephants in zoos as well as the effect of season of harvest for these types of forage. In general, thorny elaeagnus, willow oak and sweet gum were less fermentable than the intermediate fermentable tulip poplar and most fermentable were timothy hay, N&S grass and alfalfa hay, which is in line with previous research suggesting that browse species are generally less fermentable than grasses and legumes (Hummel et al. 2006a). Minimal differences in in-vitro gas production between multiple alfalfa samples and various browse leaf samples (Hummel et al. 2006b), which could be explained by the absence of browse twigs in this study whereas twigs were present in the browse samples in the present study. Furthermore, the present study showed that in-vitro fermentability of timothy hay, N&S grass and alfalfa hay varied among harvest seasons, whereas for the browse forage, harvest season did not or only affected one fermentation parameter measured. Variation in DF and ADL contents of forage correlated with variation in SCFA production and NSP only when combined with ADL. NH_3 production correlated with total CP content and with CP content corrected for NDICP content.

Currently, it is advised to feed adult African elephants in human care 1.2–1.9% DM of their bodyweight daily (Olson 2002). As hindgut fermenters, elephants rely on fermentation of fibrous forage for energy in the form of SCFA. Large differences in in-vitro SCFA production among forage were found with amounts of total SCFA produced from willow oak and sweet gum being about 30% of the amounts found for alfalfa hay and N&S grass. Per unit of DM, these types of forage would have different digestible energy contents. To balance intake levels with energy requirements, it is important to further refine the feeding guidelines beyond DM basis and move more towards feeding on digestible energy basis. To enable this refinement, data on digestibility for the range of forage commonly fed in practice are required, or alternatively, further understanding on how cell wall composition relates to digestibility. The chemical characterisation of used forage (Wood et al. 2020) allowed evaluation of key proxies to characterise cell wall content and composition being associated with in-vitro fermentability as reflected in total SCFA produced at 72 hr. Increasing dietary fibre and ADL contents of forage were associated with lower total SCFA produced, whereas NSP (calculated as dietary fibre–ADL) did not show this association. Combining information on NSP and ADL contents seemed to be a promising way to predict total SCFA and, hence, potential differences in fermentability of forage for elephants. The importance of ADL content for digestibility was also noted in other studies (e.g. Smith et al. 1972; Goto et al. 1991; Hummel et al. 2006a; He et al. 2018), which is in line with lignin being often considered as an anti-quality factor in fibrous forage. Lignification of plant cell walls enhances the structural strength and rigidity, limits water permeability and hinders pathogenic organisms (Moore and Jung 2001). Microbial degradation of lignin is limited (Smith et al. 1972; Robbins and Moen 1975) and as such lignification also renders the cell wall constituents less available for microbial fermentation. Lignification (i.e. higher ADL contents), however, explained 43.2% of the variation in total SCFA and when combined with NSP contents this increased to 63.7%, which suggests that amount of cell wall constituents other than lignin are also important and become less degradable in-vitro when the forage is richer in these two parameters. Though a better understanding how these in-vitro values translate into nutritional value in-vivo is still warranted, these findings illustrate the variability in fermentability among forage and suggest that feeding elephants on different types of forage on a DM basis could result in differences in the digestible energy provided.

Zoological institutions use forage harvested throughout the year, depending on availability. From spring to winter, various processes take place like maturation, flower and seed development, lignification and translocation of resources in plants (Raven et al. 2012a, b), which might impact the nutritional value of the forage at harvest and when fed to animals. The impact of harvest season on fermentability was greater in the present study for the grasses and legume but the effect of season was limited for browse samples. Vandermeulen et al. (2018) reported differences in in-vitro fermentability of four legume and grass species harvested in spring, summer and winter with, in general, higher total SCFA production for spring samples compared to summer and winter and no differences among seasons in the total gas production or individual SCFA. In the present study, timothy hay and alfalfa hay generally showed the lowest values for the samples harvested in summer and highest for the spring and winter sample. These samples yielded 23 and 16% less total SCFA than the average for each forage. For N&S grass, however, the summer sample yielded 7% more total SCFA than the average of the two samples, which illustrates that seasonal changes in nutritional quality (i.e. fermentability) are forage-specific. The seasonal changes in chemical composition contribute to the observed changes. For N&S grass, for example, the summer sample had considerably less lignin than the winter sample, suggesting that a lower lignification enabled a higher microbial degradation. Furthermore, the summer sample of alfalfa hay was relatively high in aNDFom, ADF, ADL and CP levels. This study's compositional values were similar for NDF (66.9%) and ADF (52.5%) contents in stems of alfalfa that were flowering (Dien et al. 2006). The ADL content in flowering alfalfa (7.1%) was lower than that in our study (12.8%) but was still higher than that of stems in the budding stage (5.5%). Furthermore, the drop in CP content from the spring to summer sample in our study is in line with the drop from budding stage to the flowering stage (Dien et al. 2006). The increase in cell wall and decrease in CP contents could be due a decreased leaf-to-stem ratio as alfalfa matures, since stems contain more lignin and ADF and less CP than leaves (Albrecht 1983). Maturation decreased degradability of stems, in particular, with values of 85% for young and 56% for matured stems when incubated with sheep rumen inoculum for 48 hr (Terry and Tilley 1964). Though changes in fermentability were noted for the grass forage used in the present study (for total SCFA 7 to 23% deviation from the average), all samples of these types of forage could still be considered well-fermentable when compared to the other (browse) forage. The

overall relatively consistent in-vitro fermentability of the selected browse forage found in this study suggests that the seasonal variations in digestible energy value would be limited in this group. This would imply that zoo nutritionists could work with one approximate value for the browse forage, whereas for the grasses and alfalfa the harvest season should be considered. For specific nutrient levels (e.g. minerals, vitamins), however, it would be of interest to have more detailed information on seasonal variations.

Forage was incubated without pre-digestion. In-vivo, however, the mastication and digestive processes in the stomach and small intestine would impact the large intestinal fermentation of the undigested fractions. For example, the majority of starch and simple sugars is likely to be digested and absorbed before forage reaches the large intestine. Using a sample containing 20% starch and 80% dried alfalfa and equine faecal inoculum, Garber et al. (2018) showed a decrease in gas production when pre-digestion was applied (121 ml/g DM) compared to when it was not applied (209 ml/g DM). The forage in the present study was low in starch (0.4–4.8%) and simple sugars (2.1–8.6%) and these digestible carbohydrates would, therefore, have a small contribution to the fermentation products with an estimated overestimation of 0.89 ml/g OM to 10.6 ml/g OM (based on the pure starch control). Next to removal of simple sugars and starch, proteins in a feedstuff would also be partly digested in-vivo. Horses have, on average, an ileal apparent dietary nitrogen digestion of 45.8% (Hendriks et al. 2012). Zeyner et al. (2015) suggested that in horses 90% of the CP minus the NDICP is digested and absorbed before reaching the large intestine. Not removing the digestible proteins from a forage prior to in-vitro incubation would result in an overestimation of protein fermentation. We found a positive correlation between feedstuff total CP content or CP corrected for NDICP content and NH_3 produced at 72 hr of incubation with slopes of 1.48 and 1.58 mg/g OM per unit of CP, respectively. Cone and Van Gelder (1999) incubated casein and starch mixtures with sheep rumen fluid and reported a slightly lower slope value of 1.43 mg/g OM. The latter study also reported a decrease in 72-hr OMCV at a rate of 2.48 ml/g OM per percentage of protein. The CP content was low in several of the present types of forage, although higher levels (≥11% CP) were found for thorny elaeagnus, alfalfa hay and N&S grass. The presence of potential digestible proteins in the forage would have lowered the OMCV values, in particular for alfalfa hay as 78 to 82% of the total CP was non-NDICP. Assuming a digestibility of 90% of these non-NDICP (Zeyner et al. 2015) in alfalfa hay and a slope of 2.48 ml/g OM for OMCV and 1.43 mg/g OM for NH_3 (Cone and Van Gelder 1999), the OMCV was underestimated by 21 to 40 ml/g OM and NH_3 was overestimated by 11.9 to 23.3 mg/g OM. It is therefore important to interpret the in-vitro fermentability results with care and use, for example, both OMCV and total SCFA as parameters to evaluate the degree of fermentability of forage. Finally, the incubation time of 72 hr is longer than the gut transit time of 38–48 hr for elephants fed alfalfa hay (Foose 1982). The long incubation time is commonly applied to enable modelling of fermentation kinetics (e.g. Bosch et al. 2008). For accurate curve fitting and calculation of kinetic parameters, it is required that the asymptote of gas production is reached for each substrate. It appeared that the forage was fermented gradually and even 72 hr was insufficient to reach an asymptote. Curve fitting was therefore not possible, though the presented gas production curves illustrate differences in kinetics between the types of forage.

Conclusion

The vulnerable status of free-ranging African elephants has prompted a surge in research to better understand their nutrition. Gastrointestinal issues, foot lesions, skin diseases, poor reproduction and elevated body condition scores are primary concerns for human-managed African elephants (Edwards et al. 2019). Although research and management changes have helped to address these concerns (Brown et al. 2019), knowing more about the fermentation of the forage fed to elephants in human care is a vital area of research. Most zoological diet programmes focus on the nutrients within each type of forage without noting the species-specific or harvest season forage digestibility differences that impact these nutrients. To develop accurate feeding programs, more research into the true nutrient digestibility of available forage is needed.

The overall difference in chemical composition among plant species noted in the current research had an effect on all the in-vitro fermentation parameters. The fermentation parameters indicated that grass and legume species had considerably higher values than browse species. To balance intake levels with energy requirements, it is important to further refine the feeding guidelines beyond DM basis and move more towards feeding on digestible energy basis taking into account potential profound differences in fermentability among forage in-vivo. Season was most important for timothy hay, N&S grass and alfalfa hay when evaluating the feed value, whereas for the selected browse forage, seasonal variations in fermentability were limited. This would imply that zoo nutritionists could work with one approximate value for the browse forage, but for the grasses and alfalfa the harvest season should be considered. A negative correlation between DF and ADL and SCFA production was found. NSP did not correlate with SCFA production, but the combination of NSP and ADL contents seemed to be a promising way to predict total SCFA and, hence, digestibility of forage for elephants. Overall, it is recommended to consider the appropriate species and seasonal fermentative capacity when including forage in diet programmes as seasonal diets may be most appropriate in order to maintain proper gastrointestinal health and body condition scores.

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