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ORIGINAL ARTICLE

Black rhinoceros (*Diceros bicornis*) and domestic horse (*Equus caballus*) hindgut microflora demonstrate similar fermentation responses to grape seed extract supplementation *in vitro*

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Summary

The domestic horse is used as a nutritional model for rhinoceros maintained under human care. The validity of this model for browsing rhinoceros has been questioned due to high prevalence of iron overload disorder (IOD) in captive black rhinoceros (*Diceros bicornis*), which is associated with high morbidity and mortality. Iron chelators, such as tannins, are under investigation as dietary supplements to ameliorate or prevent IOD in prone species. Polyphenolic compounds variably affect microbial fermentation, so the first objective of this experiment was to evaluate the effects of grape seed extract (GSE; a concentrated source of condensed tannins; CT) on black rhinoceros hindgut fermentation. Equine nutrition knowledge is used to assess supplements for rhinoceros; therefore, the second objective was to evaluate the domestic horse model for black rhinoceros fermentation and compare fermentation responses to GSE using a continuous single-flow *in vitro* culture system. Two replicated continuous culture experiments were conducted using horse and black rhinoceros faeces as inoculum sources comparing four diets with increasing GSE inclusion (0.0%, 1.3%, 2.7% and 4.0% of diet dry matter). Diet and GSE polyphenolic compositions were determined, and sodium sulphite effect on neutral detergent fibre extraction of CT-containing forages was tested. Increasing GSE inclusion stimulated microbial growth and fermentation, and proportionally increased diet CT concentration and iron-binding capacity. Horse and black rhinoceros hindgut microflora nutrient digestibility and fermentation responses to GSE did not differ, and results supported equine fermentation as an adequate model for microbial fermentation in the black rhinoceros. Interpretation of these results is limited to hindgut fermentation and further research is needed to compare foregut digestibility and nutrient absorption between these two species. Supplementation of GSE in black rhinoceros diets up to 4% is unlikely to adversely affect hindgut nutrient digestibility or microbial viability and fermentation.

Keywords condensed tannins, iron chelator, iron overload disorder, hindgut fermentation, proanthocyanidins, sodium sulphite

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Introduction

As large hindgut fermenting herbivores, the gastrointestinal anatomy of rhinoceros most resembles that of equids (Clemens and Maloiy, 1982; Endo et al., 1999; Stevens and Hume, 2004). The domestic horse (*Equus caballus*) is typically used as a nutritional model for all rhinoceros species (Oftedal et al., 1996; Dierenfeld, 1999; Miller, 2003). Fermentation products (volatile fatty acids; VFAs) significantly contribute to overall energy metabolism in these species (Glinsky et al., 1976; Harris, 1997; Santos et al., 2011), yet, to our knowledge, hindgut fermentation has never been compared between browsing rhinoceros (i.e. black rhinoceros; *Diceros bicornis*) and horses. Comparisons of hindgut fermentation capabilities may help explain differences observed in digestibility of similar diets between the two species, *in vivo* (Clauss et al., 2006a,b; Clauss et al., 2007a). Fundamental differences in digestive physiology between grazing and browsing species suggest fermentation may differ (Clauss et al., 2005a; Hummel et al., 2006). This hypothesis is supported by gas production differences measured between black and white rhinoceros *in vitro* faecal fermentation (Steuer et al., 2010). Our understanding of equine nutrition is often used to assess diets or supplements for rhinoceros; therefore, evaluating

the use of a domestic horse model for black rhinoceros fermentation is crucial.

The adequacy of the equine model has been confirmed for grazing rhinoceros species [(African white (*Ceratotherium simum*); Frape et al., 1982) and Indian rhinoceros (*Rhinoceros unicornis)*; Clauss et al., 2005b)]. However, inadequacies of the horse model have been reported for elephants (Oftedal et al., 1996); and differences in mineral nutrition between tapirs (a browsing herbivore) and horses have been measured (Clauss et al., 2009), similar to differences demonstrated between black rhinoceros and horses (Clauss et al., 2007a).

Excessive iron accumulation is observed in more than 75% of tested captive black rhinoceros and is associated with high morbidity and mortality (Molenaar et al., 2008; Paglia and Tsu, 2012). The high prevalence and severe health consequences of iron overload disorder (IOD) in captive black rhinoceros suggests a need to re-evaluate the domestic horse model for browsing rhinoceros nutrition.

To ensure the health of current and future populations of black rhinoceros managed under human care, feasible solutions and prevention strategies for IOD must be established. Nutritionists initially addressed this problem by removing supplemental iron sources and food items especially high in iron or nutrients that increase iron bioavailability. Practical limitations and availability of low-iron ingredients make it difficult to achieve recommended dietary iron concentrations below 100 ppm (Clauss et al., 2012). Therefore, the International Workshop on Iron Overload Disorder (2011) prioritized and encouraged research to identify, characterize and make recommendations of use for compounds that sequester dietary iron.

Polyphenolic compounds, such as condensed tannins (CT), are well-documented iron chelators found in substantial concentrations in natural diets of many wild animals, including browsing rhinoceros. In contrast, most diets fed in zoological institutions contain low to negligible CT concentrations (Clauss, 2003), provided through intermittent and opportunistic browse supplementation (Helary et al., 2012). This discrepancy and high incidence of associated diseases reported in iron-sensitive species confirms the importance of researching CT supplementation in captive diets when diets cannot be formulated with low iron concentrations.

Current experimental evidence supporting benefits of CT inclusion in exotic animal diets is scarce. Commercially available sources have been tested (Clauss et al., 2007b; Lavin et al., 2010), but due to prohibitive cost and ecological considerations, alternative sources must be considered. By-products of grape production industries, such as grape seed extract (GSE), are economical sources of concentrated polyphenolic compounds (Ky et al., 2014) and have been suggested as potential iron-chelating food ingredients for iron-sensitive species (Gaffney et al., 2004; Clauss et al., 2006b). Grape seed extract was evaluated in this study for potential application in black rhinoceros diets as an iron chelator.

It is important to remember the term 'tannin' and even more specific terms such as 'condensed tannin' cannot be universally applied and compared due to chemical complexity of these large polyphenolic classes. This CT diversity complicates analysis and quantification techniques. Several methods have been developed, and each has limitations that have been discussed in several reviews (Makkar et al., 1999; Scho et al., 2001; Pérez-Jiménez et al., 2009; Dai and Mumper, 2010). In this study, three quantitative methods based on different functional or structural CT properties and high-performance liquid chromatography (HPLC) were used and compared to quantify and characterize GSE CT as well as highlight the variation among results from different methods.

Tannins may influence microbial fermentation and outcomes appear to depend on polyphenolic composition and concentration (Tebib et al., 1996; Martin-Carron and Goni, 1998). Historically, tannins have been regarded as microbial growth inhibitors (Min et al., 2006; Patra and Saxena, 2011), yet there are reports of increased caecal microbial diversity in chicks (Viveros et al., 2011) and VFA production in rats (Bravo et al., 1994; Tebib et al., 1996) when CT were supplemented in the diet. However, there was no increase in faecal VFAs when CT were fed at 1.5% of dry matter (DM) to black rhinoceros (Clauss et al., 2007b). This influence makes it of interest to determine CT supplement effects on black rhinoceros microbial fermentation.

The objectives of this experiment were to compare fermentation characteristics and nutrient digestibility between the black rhinoceros and domestic horse, and to examine GSE effects on fermentation parameters and diet iron-binding potential. We hypothesized fermentation characteristics and response patterns to GSE inclusion would differ between the horse and black rhinoceros due to different physiological adaptations to contrasting natural diets of grass and browse, respectively (Hummel et al., 2006), thus influencing hindgut microflora composition. We also hypothesized diet iron-binding capacity would increase with increasing GSE inclusion.

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Methods

Experimental design

Two replicated continuous culture experiments were conducted using domestic horse and black rhinoceros faeces as inoculum to compare four diets with increasing GSE inclusion. This experiment was conducted as a randomized complete block with a two by four factorial design to analyse the effects of inoculum source (species; horse vs. rhino) and GSE inclusion (treatment; Control, Low, Mid or High). Fermenter was considered the experimental unit for all analyses. Experimental replication (continuous culture run) was the blocking variable.

Animals and faecal collection

Two adults (one male, one female, 9 years of age) and one juvenile male (3 years of age) East African black rhinoceros (*Diceros bicornis michaeli*), housed at the Saint Louis Zoo (Saint Louis, MO, USA), were fed their normal diet (Control diet; Table 1); however, browse and enrichment food items containing polyphenolics were removed for the 14 days prior to faecal collection. The Saint Louis Zoo Animal Biomaterials Sample Committee approved the protocol and collection of faeces for this experiment. Three adult (ages ranged 15–23 years) female quarter horses (*Equus caballus*), housed at the University of Missouri Equine Teaching and Research Farm (Columbia, MO, USA), were transitioned to the same control diet (Table 1) over a 5-day period and were adapted to the diet for 14 days prior to faecal collection. The University of Missouri Animal Care and Use Committee approved the use of horses for this experiment. Hay fed to horses and rhinoceros and used for both continuous culture experiments was from the same source and lot. Following the 14-day adaptation periods, total faeces were collected within 30 min of excretion and the outer layer was removed to prevent contamination. Faeces were mixed with a 1:3 glycerol-McDougall's Buffer solution (Luchini et al., 1996) at 20% (vol/wt), flushed with CO_2 and immediately frozen on dry ice for transportation. Samples were stored at −20 °C until use.

Table 1 Continuous culture experimental control diet and grape seed extract (GSE) treatment concentrations

Diet Ingredients	Per cent of control diet* (DM basis)
Orchard grass/alfalfa hay	83.05
High fibre pellets†	15.96
Kale	0.48
Carrots	0.41
Apple	0.10
Treatment	**GSE‡ inclusion level (per cent of diet DM)**
Control	0.00
Low	1.33
Mid	2.66
High	4.00

Diet ingredients were dried at 95 °C, ground to 2 mm and mixed prior to GSE inclusion. GSE replaced total diet.
*Ingredient composition presented on a dry matter (DM) basis.
†High fibre pellets (ADF-25, Mazuri® Exotic Animal Nutrition, PMI Nutrition, Saint Louis, Missouri).
‡GSE (ActiVin®, San Joaquin Valley Concentrate).

Continuous culture procedure and analyses

Inoculum preparation

Prior to inoculating the continuous culture, faecal samples were thawed and pre-incubated (Luchini et al., 1996). Equal proportions of faeces from each animal, within species, were combined, blended with equal parts (wt:v) of an anaerobic pre-incubation media (Table 2) and incubated at 39 °C for 6 h.

Experiment conditions

Twenty-four single-flow continuous culture fermenter vessels (Nalgene, Rochester NY, USA) were

Table 2 Pre-incubation media composition

Component	Amount, ml/l
Mineral solution A*	330.00
Mineral solution B†	330.00
Water soluble vitamin solution‡	25.00
Folate–biotin solution§	5.00
Riboflavin solution¶	5.00
Soluble carbohydrate solution**	50.00
Pectin solution††	100.00
Short-chain fatty acid solution‡‡	10.00
Mercaptoethanol	0.16
Cysteine-HCl $H_2 0$	0.50
Urea solution§§	10.00

Adapted from methods described by Sunvold et al. (1995).
*Composition (g/l): NaCl, 5.4; $(NH_4)_2SO_4$, 5.4; KH_2PO_4, 2.7; $MgCl_2$ $6H_2O$, 0.12; $CaCl_2$ $2H_2O$, 0.18; $MnCl_2$ $4H_2O$, 0.06; CoCl $6H_2O$, 0.06.
†Composition: K_2HPO_4, 2.7 g/l.
‡Composition (mg/l): thiamin, 92.0; pantothenic acid, 9.2; niacin, 99.5; pyridoxine, 82; p-aminobenzoic acid, 5; vitamin B-12, 0.01.
§Composition (mg/l): folic acid, 8.0; biotin, 0.02; $(NH_4)_2CO_3$, 100.
¶Composition: riboflavin, 10 mg/l in 5 mM HEPES.
**Composition (g/l): maltose, 13.5; glucose, 6.7; sucrose, 6.7; soluble potato starch, 50.0.
††Composition: citrus pectin, 26.5 g/l of 70 °C mineral solution A*.
‡‡Composition (ml/l): glacial acetic acid, 170; propionic acid, 60; *n*-butyric acid, 40; isobutyric acid, 10; *n*-valeric acid, 10; isovaleric acid, 10; DL-α-methylbutyrate, 10. Brought to volume then adjusted to pH 7.0 with 6 N NaOH.
§§Composition: $CO(NH_2)_2$, 166.62 g/l.

inoculated (12 per species) with a 1:3 (v:v) mixture of pre-incubated and strained faecal solution and 39 °C anaerobic continuous culture buffer solution, adapted from McDougall's artificial saliva (McDougall, 1948) containing 250 mg cysteine-HCl/l. Inoculum was added to each fermenter up to the effluent outflow port (1460 ml). Fermenters were continually flushed with CO_2, stirred with magnetic stir plates and incubated in a 39 °C water bath using thermostatically controlled heaters. The continuous culture buffer solution was continuously added to the fermenter containers using calibrated peristaltic pumps (Masterflex model 7520-10, Cole Palmer Instrument, Chicago, IL, USA) that maintained a constant dilution rate of 3.8% per h. Effluent was collected in vessels immersed in an ice bath.

Diets

The control diet was based on average composition of black rhinoceros diets at the Saint Louis Zoo (Table 1). Diet ingredients were ground to pass through a 2-mm screen and mixed prior to GSE inclusion. Dietary treatments (n = 6 fermenters per treatment per replicate) consisted of four GSE (ActiVin®, San Joaquin Valley Concentrate, Fresno, CA, USA) concentrations (0.0, 1.3, 2.7 and 4.0% of DM) added to the basal diet (Table 1). The four GSE concentrations were chosen to be representative of free-ranging black rhinoceros dietary CT concentrations (Helary et al., 2012). Dietary nutrient composition was similar among all treatments and averaged 94.1% DM, 91.2% organic matter (OM), 20.5% crude protein (CP), 42.2% neutral detergent fibre (NDF), 27.5% acid detergent fibre (ADF) and 247.7 ppm iron. Thirty-five g of diet was fed to the cultures per day, equally split between two feedings at 12-h intervals.

Sampling

Experiments were conducted over 8-day periods with five adaptation days and three sampling days. Fermenter pH was measured, and 10-ml samples were taken and acidified with 6 M HCl at 0, 2, 4, 6 and 8 h relative to feeding and stored at −20 °C until analysis for VFA and ammonia (NH_3) concentration. Total effluent contents were collected over 24 h of each sampling day and stored at 4 °C until further analysis. All samples were composited by fermenter over the 3 sampling days. At the end of each experiment, fermenter contents were blended, strained through four layers of cheese cloth and stored at 4 °C until analysis.

Laboratory analysis

Blended fermenter contents were centrifuged at 1000 × g for 5 min at 4 °C to remove feed particles. Supernatant was recentrifuged at 27 000 × g for 30 min, and the resulting pellet, containing bacteria, was transferred to a plastic container. Effluent contents were thoroughly mixed, and a 600-ml subsample was collected. Effluent samples and fermenter bacteria pellets were lyophilised and ground using a mortar and pestle.

Diet and effluent samples were analysed for DM by drying at 105 °C for 24 h, OM (AOAC, 2012, 934.01) and total N by combustion analysis (vario Macro Cube, Elementar Americas, Mt. Laurel, NJ, USA; AOAC, 2012, 990.03). Due to a lack of scientific consensus on sodium sulphite (SS) inclusion for detergent fibre determination in CT-containing samples (Pagán et al., 2009; Gomes et al., 2012), the effect of SS on sample NDF and ADF concentration was tested. Diet and effluent samples were analysed for NDF following the methods of Van Soest et al. (1991), both with and without addition of SS, followed by sequential ADF determination using an ANKOM^{200} Fiber Analyzer (ANKOM Technology, Macedon, NY, USA). Values for NDF and ADF, as well as fibre digestibility (digestibility coefficient = [fibre intake, g/days−(effluent DM flow, g/days × effluent % fibre)]/fibre intake, g/days × 100), were compared between the two analysis methods. Effluent and bacteria samples were analysed for purine (Zinn and Owens, 1986), and microbial nitrogen content, microbial efficiency (MOEFF; g microbial nitrogen outflow/kg OM truly digested) and microbial OM (OM_m) production per day were calculated. Fermenter samples taken at multiple time points throughout the experiment were compiled by hour, across the three sampling days. These samples were analysed spectrophotometrically (Evolution 201, Thermo Scientific, Waltham, MA) for NH_3 concentration (Broderick and Kang, 1980), and VFA concentrations were measured using gas chromatography (Model 430, Varian, Palo Alto, CA, USA; Galyean and May, 2010).

Tannin analyses

Diet, effluent and GSE samples were analysed for CT using the protein precipitable phenolics method (PPP) and the acid butanol assay. In the acid butanol assay (Hagerman, 2011), the interflavan bonds between subunits of the flavan-3-ol CT polymers were oxidatively cleaved in hot, acidic alcohol to produce coloured anthocyanidins, which were read spectrophotometrically. The PPP method quantified CT by exploiting the ability to bind and precipitate a standard protein. The method of Hagerman and Butler (1978) involved formation of a protein–tannin complex between the tannin-containing solution and

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bovine serum albumin. Condensed tannins were purified from High diet and GSE samples with Sephadex LH-20 (Sigma Aldrich, Saint Louis, MO, USA) using methods adapted from Strumeyer and Malin (1975) and Naumann et al. (2014) and were used as internally derived standards in both the acid butanol and PPP assays.

Diet and GSE samples were also analysed for iron-binding capacity following the methods of Brune et al. (1991) using tannic acid and (+)-catechin hydrate as standards. Extracted phenolic compounds (not specific to CT) react with iron and form a colour that is read spectrophotometrically at two wavelengths. The absorbencies correspond to formation of iron-galloyl complexes and iron-catechol complexes. Thus, this assay not only quantified the ability of the diet to bind iron but also allowed limited tannin structural characterization.

Purified diet and GSE CT samples were characterized by HPLC analysis for anthocyanidins. Condensed tannins were depolymerized into anthocyanidin monomers. The techniques utilized did not allow degree of polymerization estimation but determined anthocyanidin composition in CT samples. Sample solutions were prepared at 25 mg/ml of 95% butanol 5% HCl and allowed to react for 45 min in a boiling water bath. Anthocyanidin monomer analysis was conducted on the acid butanol reaction products using HPLC analysis controlled by CHROMELEON™ software (Thermo Scientific™ Dionex™, Sunnyvale, CA, USA). A 3.0 mm × 150 mm, 2.6 μm Thermo Scientific™ Accucore™ C_{18} column was used. Prior to injection, the 30 °C column was equilibrated for 5 min with 5% mobile phase B (1 ml/l trifluroacetic acid in acetonitrile). Five microlitres of each sample was injected, and separation was achieved with a mobile phase gradient, increasing mobile phase B from 5% at 0 min to 55% B at 20 min. The eluate was analysed through UV spectrophotometric detection at 280 nm for gallic acid and 550 nm for all other anthocyanidin monomers. Peaks were identified by comparing the retention time and spectra with those of commercially prepared cyanidin, delphinidin and pelargonidin standards, and peak areas were calculated with CHROMELEON™ software.

Statistical analysis

All statistical analyses were performed using SAS® version 9.4 (SAS Institute, Cary, NC, USA). True nutrient digestibility, OM_m production and MOEFF analyses were conducted using the GLM procedure. The MIXED procedure was used to analyse VFA, NH_3 and pH data using hour as a repeated measure, with fermenter as the subject and a compound symmetry covariance structure. When the *F*-test was statistically significant (a value of $p \leq 0.05$), means separation was performed using Fisher's least significant difference. Linear, quadratic and cubic contrasts were tested for the effect of GSE treatment.

Results

Tannin analyses

Condensed tannins by protein precipitable phenolics

Protein precipitable CT data are presented in Fig. 1. Results from both continuous culture replications did not differ, and data were averaged within treatment. The High diet had the greatest CT concentration (26.26 mg CT/g DM) and Control had the lowest, yet still detectable concentrations (0.7 mg CT/g DM). No detectable levels of CT were measured in any effluent sample from any treatment for either experiment. Pure GSE contained CT concentration of 636.60 ± 44.80 mg CT/g DM.

Condensed tannins by acid butanol

Proanthocyanidins (PA, i.e. condensed tannin) were not detected in any sample residue following three extractions (Unextractable PA, Table 3), indicating that GSE tannins and those intrinsic in the control diet were readily extractable in aqueous solutions. Extractable PA concentration in the diets increased with increasing GSE inclusion, values for each treatment did not differ between replications, and data were averaged within treatment (Table 3). Although no GSE was supplemented in the control diet, PAs were detected (3.31 mg/g DM), which demonstrated low inherent tannin concentrations in the control diet.

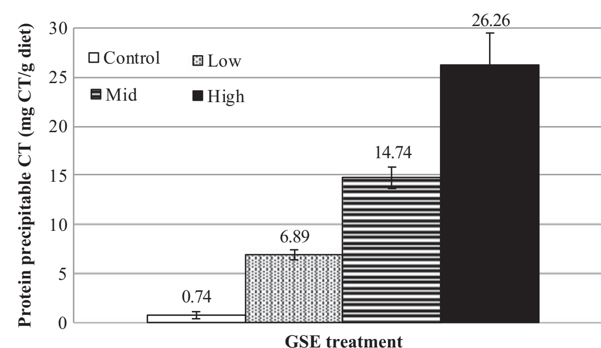

Fig. 1 Protein precipitable condensed tannins (CT) concentration, mg per g of continuous culture diet dry matter. Dietary treatments consisted of Control = basal continuous culture diet; Low = basal diet + 1.33% grape seed extract (GSE); Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE.

Table 3 Extractable and unextractable proanthocyanidin concentration (mg condensed tannin; CT/g dry matter; DM) in continuous culture diets, as determined by the acid butanol assay

Treatment*	mg extractable‡ CT/g	SD†	mg unextractable‡ CT/g
Control	3.31	0.66	0.00
Low	24.36	5.67	0.00
Mid	45.28	8.39	0.00
High	60.32	4.39	0.00

*Grape seed extract (GSE) treatment: Control = basal continuous culture diet; Low = basal diet + 1.33% GSE; Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE.
†Sample standard deviation (six replicates per treatment).
‡Extractable CT = CTs measured in the supernatant after samples were extracted three times in 70% acetone. Unextractable CT = CTs remaining in the sample after three acetone extractions.

Iron-binding capacity

Results from both continuous culture diets were averaged within treatment, and iron-binding capacity data are presented in Fig. 2. Samples demonstrated large differences between tannic acid equivalents (TAE) and (+)-catechin equivalents (CE), and the magnitude of this difference increased as GSE inclusion increased. Pure GSE had much stronger iron-binding capacity and demonstrated greater CE (1267.06 ± 45.78 CE/g DM) compared to TAE (413.85 ± 12.76 TAE/g DM).

Anthocyanidins

Purified High diet and GSE CT anthocyanidin compositions are presented in Table 4. Cyanidin:delphinidin ratio and proportion of gallated monomer units were lower in High CT compared to GSE CT (0.47 vs. 0.60),

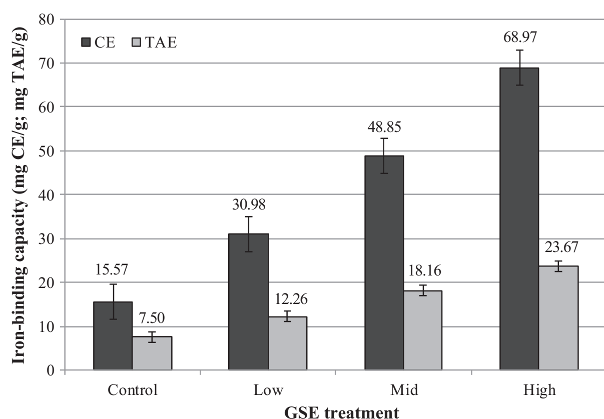

Fig. 2 Continuous culture diet iron-binding capacity. (+)-Catechin equivalents (CE) and tannic acid equivalents (TAE) per g dry matter are presented for both samples. Dietary treatments consisted of Control = basal continuous culture diet; Low = basal diet + 1.33% grape seed extract (GSE); Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE.

Table 4 High-performance liquid chromatography (HPLC) analysis of anthocyanidin monomer composition in continuous culture (CC) diet and grape seed extract (GSE) purified condensed tannins (CT)

Sample	Cyanidin:delphinidin‡	Proportion gallated§
High CC diet CT*	8.5:1	0.47
GSE†	14.0:1	0.60

*CT were purified from the High CC diet (Control diet + 4.00% GSE) prior to preparation for HPLC analysis.
†GSE (ActiVin®, San Joaquin Valley Concentrate) CT were purified prior to preparation for HPLC analysis.
‡Proportion of cyanidin monomers compared to delphinidin monomers.
§Proportion of anthocyanidin monomers with a gallic acid moiety attached.

and no pelargonidin monomers were detected in either sample.

Effect of sodium sulphite on fibre analysis of tannin-containing samples

Both diet and effluent NDF and ADF values were greater ($p \leq 0.05$) when determined without SS (Table 5). As there is no reason to believe SS is solubilizing cell wall components, the lower values are likely more accurate. Neutral detergent fibre digestibility was greater ($p = 0.001$) and ADF digestibility tended to be greater ($p = 0.09$) when calculated using SS-included NDF and ADF values (Table 5). No interactions between SS inclusion and dietary treatments were measured in any of the three data sets ($p \geq 0.15$). All NDF and ADF results presented in this article were calculated using the detergent fibre analysis procedures of Van Soest et al. (1991) with the addition of α-amylase and SS because results suggest fibre values determined using SS are more accurate.

Continuous culture results

Effect of continuous culture run (i.e. replication) was tested for all variables. Unless otherwise discussed, there was no replication by treatment or species interaction ($p > 0.05$), indicating that variables responded similarly in each experimental replication.

Digestibility and microbial measurements

Nutrient digestibility and microbial measurements are presented in Table 6. There were no significant species by treatment interactions measured for these variables ($p \geq 0.15$). Grape seed extract inclusion quadratically affected OM and ADF digestibility ($p \leq 0.02$) and tended to decrease NDF digestibility ($p = 0.06$). Increasing GSE inclusion linearly increased OM_m

Table 5 Effect of sodium sulphite (SS) on sample fibre concentration and digestibility calculations

	NDF*				ADF†			
Sample type	SS‡	No SS‡	SEM	p-value	SS‡	No SS‡	SEM	p-value
Diet	42.23[b]	44.24[a]	0.342	0.007	27.59[b]	28.20[a]	0.224	0.054
Effluent	20.44[b]	23.35[a]	0.270	< 0.001	13.56[b]	14.47[a]	0.184	< 0.001
Digestibility§, %	40.77[a]	34.79[b]	1.318	0.001	39.51	36.18	1.393	0.095

*Neutral detergent fibre (NDF) concentration (per cent of dry matter).
†Acid detergent fibre (ADF) concentration (per cent of dry matter).
‡Fibre analysis was performed with (SS) or without (No SS) sodium sulphite during the NDF procedure.
§True NDF or ADF digestibility in two replicated continuous culture experiments.
[a,b]Means within effect (% NDF or % ADF) with no superscripts in common, within the same row, are statistically different ($p \leq 0.05$).

Table 6 Effects of grape seed extract (GSE) treatment, species inoculum and their interaction on continuous culture nutrient digestibility and microbial production measures

	GSE Treatment* Effect						Species† Effect				Interaction‡ Effect
Nutrient Digestibility§, %	Control	Low	Mid	High	SEM	Treatment p-value	Rhino	Horse	SEM	Species p-value	Treatment × Species p-value
OM	46.55[b]	42.46[b]	48.36[b]	55.85[a]	0.025	0.003	46.95	49.66	1.553	0.240	0.891
CP	55.57	48.24	49.72	52.42	0.034	0.358	49.43	53.85	2.342	0.176	0.666
NDF	46.32	38.53	37.51	41.32	0.027	0.063	40.42	40.99	1.823	0.821	0.226
ADF	44.91[a]	36.83[b]	35.61[b]	41.25[ab]	0.027	0.037	39.66	39.10	1.765	0.820	0.148
Microbial Measures											
MOEFF¶	20.88	22.21	23.73	23.70	1.173	0.161	23.76	21.68	0.794	0.064	0.342
OM_m**, g/day	2.75[b]	2.86[b]	3.72[a]	4.29[a]	0.284	0.001	3.66	3.17	0.190	0.067	0.861

*GSE treatment: Control = basal continuous culture diet; Low = basal diet + 1.33% GSE; Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE.
†Fermenters were inoculated with faecal microbial populations from either black rhinoceros (Rhino) or domestic horses (Horse).
‡Experiment was designed as a 2 × 4 factorial to test the interaction of GSE treatment and species. No significant interactions were measured for any variables described here ($p \leq 0.05$).
§Organic matter (OM), crude protein (CP) and neutral and acid detergent fibre (NDF, ADF) true digestibility was measured.
¶Microbial efficiency (MOEFF); g microbial nitrogen outflow/kg OM truly digested.
**g of microbial organic matter produced per day.
[a,b]Means within effect (treatment or species) with no superscripts in common within the same row are statistically different ($p \leq 0.05$).

production (g/days, $p < 0.0001$) but did not affect MOEFF ($p = 0.16$). Nutrient digestibility did not differ between species ($p \geq 0.18$). Microbial production measures (MOEFF and OM_m) tended to be lower in horse fermenters compared to rhino fermenters ($p \leq 0.07$).

Fermentation characteristics

Sampling time point (h) and most interaction effects including hour were significant ($p < 0.0001$). This result was expected because pH, VFA and NH_3 concentrations are functions of diet fermentation, which changes with time. Therefore, the effect of hour will not be discussed. A treatment by species interaction was measured for NH_3 ($p = 0.001$, Fig. 3). Both horse and rhino fermenter NH_3 concentrations decreased linearly with increasing GSE inclusion ($p \leq 0.05$); however, species differences in the linear decrease slope resulted in detection of a significant species by GSE interaction. Within treatment, horse fermenters had at least 2.3 times greater NH_3 concentration at every time point measured ($p < 0.0001$; Fig. 3).

Overall, fermenter pH values did not vary extensively throughout the experiment (range: 6.06 – 7.04), yet a species by hour interaction was detected ($p < 0.0001$). At all hour points, horse fermenters had greater pH than rhino fermenters ($p < 0.0001$; 6.83 ± 0.09 vs. 6.68 ± 0.17). Dietary treatment did not affect fermenter pH values ($p = 0.73$). There were no significant interactions between species and treatment for VFA concentrations ($p \geq 0.10$). Therefore, main effects of treatment and species on VFA concentrations are presented in Table 7.

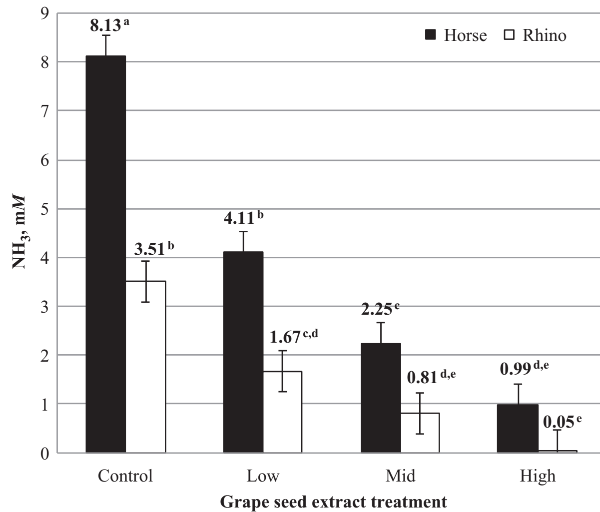

Fig. 3 Effects of continuous culture dietary treatment and inoculum species on overall fermenter ammonia (NH_3) concentrations (mM). Species by treatment interaction, p = 0.0012. a-e Means of bars with no superscripts in common are statistically different (p 0.05). Dietary treatments consisted of Control = basal continuous culture diet; Low = basal diet + 1.33% grape seed extract (GSE); Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE. Fermenters were inoculated with faecal microbial populations from either black rhinoceros (Rhino) or domestic horses (Horse).

Discussion

Tannin analyses

Three quantitative methods based on different functional or structural CT properties were used to quantify and characterize GSE and diet CT. Wild herbivore diets are most commonly assayed for CT using the acid butanol assay in field research (Wright, 1997; Kipchumba, 2002; Helary et al., 2012), and iron-binding (Ward and Hunt, 2001) and protein precipitation assays (Ward et al., 2005) have been used to quantify black rhinoceros dietary CT concentration in zoos. Comparisons across studies using different methods and even comparisons within methods must be made with caution. Inconsistent or inappropriate standards and method conditions complicate interpretation and direct comparison of results. Condensed tannins are characterized based on hydroxylation patterns, stereochemistry, functional groups, interflavan linkages and degree of polymerization (Ayres et al., 1997; Naumann et al., 2013b). Each of these chemical characteristics relates some specific functionality to the compounds. In this context, the results of all three methods can be uniquely useful for general CT characterization of the diets used in this study.

Condensed tannins by protein precipitable phenolics

The PPP assay is a functional CT quantification assay based on the ability of extracted tannins to bind and precipitate protein in a solution (Hagerman and Butler, 1978). Protein precipitation has been positively correlated with degree of CT polymerization (Hagerman, 2012; Harbertson et al., 2014; Lorenz et al., 2014). Protein precipitation capacity of the experimental diets was almost completely derived from GSE addition, thus, results suggest GSE CT had a greater degree of polymerization than that of intrinsic in the control diet. Grape seed extract contained 636 mg CT/g, and when supplemented at 4% of diet DM, the High diet was calculated to theoretically contain

Table 7 Volatile fatty acid (VFA) concentration of continuous culture fermenters

	GSE Treatment*						Species†			
Fermenter VFA measurement‡	Control	Low	Mid	High	SEM	p-value	Horse	Rhino	SEM	p-value
Ace:Pro§	2.70	2.89	2.95	2.86	0.108	0.2254	3.00[a]	2.68[b]	0.070	0.001
VFA¶ concentrations, mM										
Total	99.35[a]	92.18[a,b]	92.45[a,b]	85.05[b]	3.215	0.043	90.74	94.57	2.075	0.430
Ace	63.04	59.85	60.37	55.42	2.195	0.108	59.22	60.55	1.417	0.871
Pro	23.69[a]	20.92[b]	20.80[b]	19.44[b]	0.812	0.016	19.86[b]	22.85[a]	0.524	0.002
But	8.22	7.74	7.78	7.31	0.461	0.661	8.01	7.53	0.297	0.320
Isobut	0.79[a]	0.67[b]	0.61[b]	0.48[c]	0.030	<0.001	0.70[a]	0.58[b]	0.019	<0.001
Val	2.93[a]	2.54[b]	2.49[b]	2.12[b]	0.128	0.005	2.38[b]	2.70[a]	0.083	0.031
Isoval	0.68[a]	0.48[b]	0.40[b]	0.28[c]	0.030	<0.001	0.57[a]	0.36[b]	0.020	<0.001

There were no significant interactions between species and GSE treatment ($p \geq 0.10$).
*GSE treatment: Control = basal continuous culture diet; Low = basal diet + 1.33% GSE; Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE.
†Continuous culture fermenters were inoculated with faecal microbial populations from either black rhinoceros (Rhino) or domestic horses (Horse).
‡Fermenter samples were taken every 2 h (up to 8 h) after morning feeding. VFA measurements presented are averaged across all h.
§Acetate:propionate ratio.
¶VFAs measured: Total = Acetate (Ace) + Propionate (Pro) + Butyrate (But) + Isobutyrate (Isobut) + Valerate (Val) + Isovalerate (Isoval).
[a,b,c]Means within effect (species or treatment) with no superscripts in common within the same row are statistically different ($p \leq 0.05$).

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approximately 25 mg CT/g. Results from the PPP method measured 26 mg CT/g High diet. Protein precipitable phenolics measured in Control, Low and Mid diets also closely matched predicted CT concentrations. Although protein-binding strength increased as GSE inclusion increased, no differences were observed in CP digestibility. This result was unexpected, and caution is advised for supplementing GSE at concentrations greater than 4% due to its strong protein-binding ability determined by the PPP assay.

Condensed tannins by acid butanol

The acid butanol assay relies on detection of monomer anthocyanidin units after the CT has presumably been depolymerized. Generally, smaller polyphenolic units are more easily cleaved and result in faster and more intense colour development in the assay (Pérez-Jiménez et al., 2009). A key limitation of the acid butanol assay is the varying stability or reactivity of interflavan bonds (Scho et al., 2001). For example, interflavan bonds of quebracho tannins have low reactivity in the assay, yielding less intense colour formation at high concentrations (Rautio et al., 2007). Diets were measured to have greater CT concentrations when determined by the acid butanol assay compared to the PPP assay. Interestingly, GSE demonstrated an opposite trend. These results suggest that diet CT were readily cleaved in the acid butanol reaction and were possibly composed of smaller polymerized anthocyanidin units than GSE CT.

Iron-binding capacity

Our hypothesis that diet iron-binding capacity would increase as GSE inclusion increased was confirmed. Grape seed extract inclusion of 1.33% effectively doubled the iron-binding capacity of the diet and 4% GSE inclusion more than quadrupled the iron-binding capacity. While *in vivo* trials are needed to establish physiologically effective GSE supplementation levels, results from this study support GSE as a promising supplement to minimize available dietary iron in the intestinal lumen.

The continuous culture effluent could not be analysed for iron-binding capacity due to limited sample, so it can only be speculated how iron-binding capacity change due to fermentative digestion. Condensed tannins have been shown to be extensively metabolized by gut microflora to produce smaller molecules (Choy and Waterhouse, 2014), and results from protein precipitable phenolics analysis of the effluent in the present study support this claim. Functional group cleavage reactions (i.e. dehydroxylation, demethylation and decarboxylation) may also occur (Aura, 2008). Therefore, it is possible that following fermentation the polyphenol-chelated iron may be released into the lumen. In the context of reducing bioavailable dietary iron, this should not matter much because iron is absorbed prior to the primary fermentation site.

The iron-binding assay methodology allows not only functional conclusions (iron-binding capacity) but also some speculation on phenolic compound structure. In this assay, extracted phenolic compounds react with iron and form a colour that is read spectrophotometrically at two wavelengths. The absorbencies correspond to formation of iron-galloyl complexes (tannic acid equivalents) and iron-catechol complexes ((+)-catechin equivalents). In all samples, there were large differences between iron-binding capacity as TAE and CE. Greater iron-binding values as CE indicate that both GSE and diet samples primarily contained condensed tannins, as opposed to hydrolyzable tannins (TAE). The proportion of iron binding as TAE decreased as dietary GSE inclusion increased, indicating greater hydrolyzable tannin-like qualities in the control diet compared to GSE. Furthermore, greater catechin equivalents of Control diet than CT concentration (determined by the acid butanol assay) suggest these phenolic molecules were not small proanthocyanidins or monomeric anthocyanins, but rather other polyphenolic molecules that bound iron but did not result in colour formation when reacted with butanol HCl.

Anthocyanidins

High-performance liquid chromatography is the method of choice for anthocyanidin determination because this technique allows efficient separation, identification and quantification of anthocyanidins in CT-containing samples. Cyanidin indicates a dihydroxylated pattern in the flavonoid B-ring, and delphinidin indicates a trihydroxylation pattern. The cyanidin:delphinidin ratio of plant CT is often reported (Bate-Smith, 1975; Foo and Porter, 1980; Naumann et al., 2013a) and is related to its radical-scavenging capability (Dai and Mumper, 2010). Purified High diet CT had greater proportions of delphinidin compared to purified GSE. Increasing free hydroxyl groups, determined by greater delphinidin proportions, generally improves CT-metal ion chelation per flavonoid unit (Andjelkovic et al., 2006; Mladěnka et al., 2011). This may help explain the greater iron-binding capacity of the control diet compared to its low CT concentration, determined by PPP. In contrast, purified GSE CT had a higher proportion of gallate monomers, which may contribute to

improved iron-chelation ability (Andjelkovic et al., 2006) through increased potential for hydrogen bonding (Harbertson et al., 2014). While HPLC anthocyanidin analysis is of interest to relate CT functional properties to structural composition, the relationships are only speculative and are limited to anthocyanidin standards availability and equipment sensitivity.

All three quantitative methods confirmed linear CT concentration increased as GSE inclusion increased. No GSE was added to the control diet yet low CT concentrations were detected by all methods, indicating the control diet contained small amounts (approximately 0.3%) of inherent polyphenols. These findings suggest that tannins inherent in the control diet differed from those in GSE and may have been small polyphenolic molecules that cleaved easily in acidic alcohol and efficiently bound iron (Mladěnka et al., 2011) but lacked strong protein-binding ability (Harbertson et al., 2014). Protein precipitation capacity of the continuous culture diets was almost completely derived from GSE addition.

Microbial metabolism of polyphenolic compounds may convert large polymerized tannins into smaller, bioactive compounds that influence host health, both positively and negatively (Kemperman et al., 2010). Microbial metabolism of polyphenols is a crucial factor affecting CT bioavailability and may result in conversion of CT to potentially toxic metabolites that may be absorbed by the animal (Aura, 2008; Gross et al., 2010; Dall'Asta et al., 2012). In this experiment, effluent was assayed for intact CT using the PPP method. Degree of CT polymerization is positively correlated with its ability to precipitate protein (Harbertson et al., 2014). A lack of CT detection in the effluent suggests that both horse and rhinoceros hindgut microflora depolymerized CT in all experimental diets to a size that was too small to effectively bind protein. Future *in vivo* trials researching CT supplementation to hindgut fermenting herbivores should screen blood and urine samples for any potentially toxic metabolites.

Experimental CT concentrations reflected typical CT concentrations in the natural diets of black rhinoceros. Helary et al. (2012) analysed dietary CT concentrations in three free-ranging black rhinoceros populations (using the acid butanol assay with sorghum tannin as a standard) and found seasonal and geographical variation with averages ranging from 1.7% to 4.3% of DM. Others reported CT concentration ranges from 0.2% to 18% of diet DM (Furstenburg and van Hoven, 1994; Atkinson et al., 1997). Acid butanol results from this study (using internally derived CT standards) indicated CT concentrations of experimental diets were 0.33%, 2.44%, 4.53% and 6.03%.

Effect of sodium sulphite on fibre analysis of tannin-containing samples

Although SS is routinely used in neutral detergent fibre analysis for improved assay accuracy, its use has been questioned for high-CT samples due to variable reports on NDF and ADF concentration (Terrill et al., 2010; Gomes et al., 2012). Results appear to depend on plant type and CT concentration (Pagán et al., 2009). Terrill et al. (1994) reported that both NDF and ADF variations in CT-containing forage samples were minimized when SS was used with crucible fibre determination methods. Similar results suggested SS inclusion and sequential ADF determination should be used for CT-containing samples with the detergent analysis system (Terrill et al., 2010). In this study, forage-based diet samples with moderate levels of CT from GSE were more accurately analysed for fibre components with SS addition for NDF analysis, followed by sequential ADF analysis.

Continuous culture

Contrary to our hypothesis, the results supported equine microbial fermentation as an adequate model for microbial fermentation in black rhinoceros. The lack of species and GSE interaction effects for nutrient digestibility, MOEFF, OM_m, pH and VFA concentrations indicated that hindgut microflora fermentation in both species responded similarly to changes in dietary GSE CT concentrations, under our experimental conditions. Interpretation of these results is limited to hindgut digestion and assumes similar nutrients are reaching the hindgut in both species, which has not yet been evaluated *in vivo*.

Experimental standardization of diet, particle size and dilution rate (i.e. retention time) means the effects of test diet on microflora derived from the different species in the present study is likely real and not due to these factors, previously shown to differ between browsing and grazing herbivores, which would affect hindgut microflora populations. It is well established that diet composition (i.e. grass vs. browse) significantly affects fermentation characteristics (Hummel et al., 2006) and gut microbiota (Ley et al., 2008; David et al., 2014). Particle size influences diet digestibility (Bjorndal et al., 1990; Clauss et al., 2015), and due to differences in chewing efficiency, equids typically ingest smaller particle sizes than rhinoceros (Fritz et al., 2009). Comparisons of

total tract digestion coefficients between black rhinoceros and grazing rhinoceros species found shorter particle retention times and decreased fibre digestibility in the browsing rhinoceros species (Clauss et al., 2006a; Steuer et al., 2010). Clauss et al. (2006a) measured total tract digestion coefficients from eight black rhinoceros on three diets and compared their data to horse digestion coefficients from multiple studies using similar diets. These researchers reported lower OM and crude fibre digestion coefficients in the black rhinoceros. The current study found no species effect on nutrient digestibility by hindgut microbial populations, suggesting that the reduced OM and fibre digestibility reported by Clauss et al. (2006a) may have resulted from faster passage rates, smaller particle size or decreased foregut digestive efficiency rather than decreased microbial fermentation capacity in black rhinoceros compared to horses. A lack of species effect on total VFA production in this experiment supports this hypothesis.

Lower acetate:propionate ratios, NH_3 concentrations, pH, and greater MOEFF and OM_m in rhinoceros fermenters may indicate that the type of fermentation and microflora composition differed, but this has not been assessed through metagenomic analyses. These fermentation differences may also be a consequence of standardizing the inoculum across species per unit of faecal matter as opposed to microbial concentration, or of using faecal vs. caecal inoculum. The microbiome differs throughout different regions of the equine hindgut (Dougal et al., 2012), and in rats, *in vitro* total VFA production is greater due to using caecal contents vs. faeces as an inoculum source (Monsma and Marlett, 1995). Specifically, a longer lag-phase was observed with faecal inoculum (Monsma and Marlett, 1996). In the present study, we attempted to avoid a lag-phase effect by allowing a 6 h pre-incubation and 5-day adaptation period. Nevertheless, digestibility results from equine *in vitro* fermentation experiments utilizing faecal inoculum have adequately estimated relative *in vivo* DM digestibility within a wide range of feedstuffs (Lowman et al., 1999; Lattimer et al., 2007; Earing et al., 2010). Most importantly, it is not feasible to collect caecal contents from endangered species, such as the black rhinoceros, but faeces can be accessed without invasive techniques.

Increasing GSE inclusion appeared to stimulate microbial growth, as indicated by a linear increase in microbial OM production, decreased NH_3 concentration, increased OM digestibility and lower total VFA concentrations. Contrary to our expectations, there was no GSE effect on CP digestion, but increasing GSE inclusion linearly decreased NH_3 concentrations. Nitrogen released from dietary protein was stoichiometrically accounted for as either NH_3-N or microbial protein. Therefore, it appears that dietary nitrogen exceeded microbial requirements for the growth level obtained at a 3.8% per h dilution rate (Meng et al., 2000; Brooks et al., 2012). This hypothesis is supported by a lack of GSE effect on MOEFF, for which maximum values are determined by dilution rate (Meng et al., 1999). Although MOEFF at this dilution rate may have been maximized, increasing GSE supplementation further increased microbial OM production, suggesting that GSE tannins were stimulatory rather than inhibitory for black rhinoceros and horse hindgut microbial populations. Grape CT supplementation resulted in similar fermentative activity increases in rats (Tebib et al., 1996). In addition, broiler chicks fed grape CT concentrates had increased caecal microbial biodiversity (Viveros et al., 2011). It would be interesting to compare microbial population composition among GSE treatment levels to see whether the stimulatory growth effect was universal or specific to microbial taxonomic and/or functional groups.

In rumen *in vitro* studies, different substrates varied in how microflora partitioned fermentation derived energy, either towards microbial synthesis or into VFAs and thus gas production, which are inversely related (Blümmel et al., 1997). It was proposed that dietary compounds or feeds vary in their 'partitioning factor', meaning that given similar digestibility, some compounds result in greater nutrient partitioning towards microbial synthesis and less towards VFA and gas production. This effect was observed with saponin supplementation (Makkar et al., 1998) and our data also seem to fit this hypothesis. Grape seed extract inclusion appeared to shift fermentation characteristics along that continuum with greater partitioning towards microbial synthesis. As GSE inclusion increased, total VFA concentration generally decreased while OM_m increased, coupled with greater OM digestibility.

In conclusion, domestic horse and black rhinoceros hindgut microflora nutrient digestibility and fermentation responses to GSE did not differ. Results from this study support equine fermentation as an adequate model for microbial fermentation in the black rhinoceros. However, it is important to remember that interpretation of these results is limited to hindgut fermentation, and further research is needed to compare foregut digestibility and nutrient absorption between

these two species. Grape seed extract was found to be an effective iron chelator, and supplementation in black rhinoceros diets up to 4% of DM is unlikely to adversely affect macronutrient hindgut digestibility or microbial viability and fermentation. *In vivo* trials are needed to determine supplementation levels necessary to limit iron absorption and tissue accumulation in captive black rhinoceros.

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References

Andjelkovic, M.; Van Camp, J.; De Meulenaer, B.; Depaemelaere, G.; Socaciu, C.; Verloo, M.; Verhe, R., 2006: Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry* **98**, 23–31.

AOAC, 2012: *Official Methods of Analysis of AOAC International*. AOAC International, Gaithersburg.

Atkinson, S. J.; do Toit, J.; Topps, J., 1997: Maintenance of captive black rhinoceros (*Diceros bicornis*) on indigenous browse in Zimbabwe: nutrition and energetics. *Proceedings of the Nutrition Advisory Group*, **2**, 1–2.

Aura, A. M., 2008: Microbial metabolism of dietary phenolic compounds in the colon. *Phytochemistry Reviews* **7**, 407–429.

Ayres, M. P.; Clausen, T. P.; MacLean, S. F.; Redman, A.; Reichardt, P., 1997: Diversity of structure and antiherbivore activity in condensed tannins. *Ecology* **78**, 1696–1712.

Bate-Smith, E. C., 1975: Phytochemistry of proanthocyanidins. *Phytochemistry* **14**, 1107–1113.

Bjorndal, K. A.; Bolten, A. B.; Moore, J. E., 1990: Digestive fermentation in herbivores: effect of food particle size. *Physiological Zoology* **63**, 710–721.

Blümmel, M.; Steingass, H.; Becker, K., 1997: The relationship between in vitro gas production, in vitro microbial biomass yield and 15N incorporation and its implications for the prediction of voluntary feed intake of roughages. *The British Journal of Nutrition* **77**, 911–921.

Bravo, L.; Abia, R.; Eastwood, M. A.; Saura-Calixto, F., 1994: Degradation of polyphenols (catechin and tannic acid) in the rat intestinal tract. Effect on colonic fermentation and fecal output. *British Journal of Nutrition* **71**, 933–946.

Broderick, G. A.; Kang, J. H., 1980: Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. *Journal of Dairy Science* **63**, 64–75.

Brooks, M. A.; Harvey, R. M.; Johnson, N. F.; Kerley, M. S., 2012: Rumen degradable protein supply affects microbial efficiency in continuous culture and growth in steers. *Journal of Animal Science* **90**, 4985–4994.

Brune, M.; Hallberg, L.; Skanberg, A. B., 1991: Determination of iron-binding phenolic groups in food. *Journal of Food Science* **56**, 128–131.

Choy, Y. Y.; Waterhouse, A. L., 2014: Proanthocyanidin metabolism, a mini review. *Nutrition and Aging* **2**, 111–116.

Clauss, M., 2003: Tannins in the nutrition of wild animals- A review. Zoo Animal Nutrition Vol. II. Furth, pp. 53–89.

Clauss, M.; Froeschle, T.; Castell, J.; Hatt, J.-M.; Ortmann, S.; Streich, W. J.; Hummel, J., 2005a: Fluid and particle retention times in the black rhinoceros (*Diceros bicornis*), a large hindgut-fermenting browser. *Acta Theriologica* **50**, 367–376.

Clauss, M.; Polster, C.; Kienzle, E.; Wiesner, H.; Baumgartner, K.; von Houwald, F.; Ortmann, S.; Streich, W. J.; Dierenfeld, E. S., 2005b: Studies on digestive physiology and feed digestibilities in captive Indian rhinoceros (*Rhinoceros unicornis*). *Journal of Animal Physiology and Animal Nutrition* **89**, 229–237.

Clauss, M.; Castell, J.; Kienzle, E.; Dierenfeld, E.; Flach, E.; Behlert, O.; Ortmann, S.; Streich, W.; Hummel, J.; Hatt, J.-M., 2006a: Digestion coefficients achieved by the black rhinoceros (*Diceros bicornis*), a large browsing hindgut fermenter. *Journal of Animal Physiology and Animal Nutrition* **90**, 325–334.

Clauss, M.; Pellegrini, N.; Castell, J. C.; Kienzle, E.; Dierenfeld, E. S.; Hummel, J.; Flach, E. J.; Streich, W. J.; Hatt, J.-M., 2006b: Antioxidant status of faeces of captive black rhinoceros (*Diceros bicornis*) in relation to dietary tannin supplementation. *Journal of Veterinary Medicine* **53**, 319–322.

Clauss, M.; Castell, J. C.; Kienzle, E.; Schramel, P.; Dierenfeld, E. S.; Flach, E. J.; Behlert, O.; Streich, W. J.; Hummel, J.; Hatt, J.-M., 2007a: Mineral absorption in the black rhinoceros (*Diceros bicornis*) as compared with the domestic horse. *Journal of Animal Physiology and Animal Nutrition* **91**, 193–204.

Clauss, M.; Castell, J. C.; Kienzle, E.; Dierenfeld, E. S.; Flach, E. J.; Behlert, O.; Ortmann, S.; Streich, W. J.; Hummel, J.; Hatt, J.-M., 2007b: The influence of dietary tannin supplementation on digestive performance in captive black rhinoceros (*Diceros bicornis*). *Journal of Animal Physiology and Animal Nutrition* **91**, 449–458.

Clauss, M.; Lang-Deuerling, S.; Kienzle, E.; Medici, E. P.; Hummel, J., 2009: Mineral absorption in tapirs (*Tapirus spp.*) as compared to the domestic horse. *Journal of Animal Physiology and Animal Nutrition* **93**, 768–776.

Clauss, M.; Dierenfeld, E. S.; Klasing, K. C.; Koutsos, L.; Livingston, S.; Nielson, B.; Sullivan, K.; Valdes, E.; Ward, A., 2012: IOD in rhinos - Nutrition group report: report from the nutrition working group of the International Workshop on Iron Overload Disorder in Browsing Rhinoceros (February 2011). *Journal of Zoo and Wildlife Medicine* **43**, S108–S113.

Clauss, M.; Steuer, P.; Erlinghagen-Lückerath, K.; Kaandorp, J.; Fritz, J.; Südekum, K.-H.; Hummel, J., 2015: Faecal particle size: digestive physiology meets herbivore diversity. *Comparative Biochemistry and Physiology A* **179**, 182–191.

Clemens, E. T.; Maloiy, G. M. O., 1982: The digestive physiology of three East African herbivores: the elephant, rhinoceros and hippopotamus. *Journal of Zoology* **198**, 141–156.

Dai, J.; Mumper, R. J., 2010: Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules* **15**, 7313–7352.

Dall'Asta, M.; Calani, L.; Tedeschi, M.; Jechiu, L.; Brighenti, F.; Del Rio, D., 2012: Identification of microbial metabolites derived from in vitro fecal

fermentation of different polyphenolic food sources. *Nutrition* **28**, 197–203.

David, L. A.; Maurice, C. F.; Carmody, R. N.; Gootenberg, D. B.; Button, J. E.; Wolfe, B. E.; Ling, A. V.; Devlin, A. S.; Varma, Y.; Fischbach, M. A.; Biddinger, S. B.; Dutton, R. J.; Turnbaugh, P. J., 2014: Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**, 559–563.

Dierenfeld, E. S., 1999: Rhinoceros feeding and nutrition. In: M. E. Fowler, R. E. Miller (eds), *Zoo and Wild Animal Medicine, Current Therapy*. 4th edn. W.B. Saunders, Philadelphia, pp. 568–571.

Dougal, K.; Harris, P. A.; Edwards, A.; Pachebat, J. A.; Blackmore, T. M.; Worgan, H. J.; Newbold, C. J., 2012: A comparison of the microbiome and the metabolome of different regions of the equine hindgut. *FEMS Microbiology Ecology* **82**, 642–652.

Earing, J. E.; Cassill, B. D.; Hayes, S. H.; Vanzant, E. S.; Lawrence, L. M., 2010: Comparison of in vitro digestibility estimates using the Daisy II incubator with in vivo digestibility estimates in horses. *Journal of Animal Science* **88**, 3954–3963.

Endo, H.; Morigaki, T.; Fujisawa, M.; Yamagiwa, D.; Sasaki, M.; Kimura, J., 1999: Morphology of the intestinal tract in the white rhinoceros (*Ceratotherium simum*). *Anatomia, Histologia, Embryologia* **28**, 303–305.

Foo, L. Y.; Porter, L. J., 1980: The phytochemistry of proanthocyanidin polymers. *Phytochemistry* **19**, 1747–1754.

Frape, D. L.; Tuck, M. G.; Sutcliffe, N. H.; Jones, D. B., 1982: The use of inert markers in the measurement of the digestibility of cubed concentrates and of hay given in several proportions to the pony, horse and white rhinoceros (*Diceros simus*). *Comparative Biochemistry and Physiology A* **72**, 77–83.

Fritz, J.; Hummel, J.; Kienzle, E.; Arnold, C.; Nunn, C.; Clauss, M., 2009: Comparative chewing efficiency in mammalian herbivores. *Oikos* **118**, 1623–1632.

Furstenburg, D.; van Hoven, W., 1994: Condensed tannin as anti-defoliate agent against browsing by giraffe (*Giraffa camelopardalis*) in the Kruger National Park. *Comparative Biochemistry and Physiology A* **107**, 425–431.

Gaffney, S.; Williams, V.; Flynn, P.; Carlino, R.; Mowry, C.; Dierenfeld, E. S.; Babb, C.; Fan, J.; Tramontano, W. A., 2004: Tannin/Polyphenol effects on iron solubilization in vitro. *Bios* **75**, 43–52.

Galyean, M.; May, T., 2010: *Laboratory Procedures in Animal Nutrition Research*. 14th edn. Texas Tech University, Lubbock.

Glinsky, M. J.; Smith, R. M.; Spires, H. R.; Davis, C. L., 1976: Measurement of volatile fatty acid production rates in the cecum on the pony. *Journal of Animal Science* **42**, 1465–1470.

Gomes, D. Í.; Detmann, E.; Filho, S. D. C. V.; Mezzomo, R.; de Paiva Souza, N. K.; de Queiroz, A. C.; da Detmann, K. S. C., 2012: Evaluation of sodium sulfite and protein correction in analyses of fibrous compounds in tropical forages. *Revista Brasileira de Zootecnia* **41**, 225–231.

Gross, G.; Jacobs, D. M.; Peters, S.; Possemiers, S.; van Duynhoven, J.; Vaughn, E. E.; van de Wiele, T., 2010: In vitro bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. *Journal of Agricultural and Food Chemistry* **58**, 10236–10246.

Hagerman, A. E., 2011: *Method for Acid Butanol Assay of Dried Fig Samples. Tannin Handbook*. Miami University, Oxford. http://www.users.miamioh.edu/hagermae/.

Hagerman, A. E., 2012: Fifty years of polyphenol-protein complexes. In: V. Cheynier, P. Garni-Manchado, S. Quideau (eds.), *Recent Advances in Polyphenol Research*. 3rd edition. Wiley-Blackwell, Chichester, UK, pp. 71–97.

Hagerman, A. E.; Butler, L. G., 1978: Protein precipitation method for the quantitative determination of tannins. *Journal of Agricultural and Food Chemistry* **26**, 809–812.

Harbertson, J. F.; Kilmister, R. L.; Kelm, M. A.; Downey, M. O., 2014: Impact of condensed tannin size as individual and mixed polymers on bovine serum albumin precipitation. *Food Chemistry* **160**, 16–21.

Harris, P., 1997: Energy sources and requirements of the exercising horse. *Annual Review of Nutrition* **17**, 185–210.

Helary, S. F.; Shaw, J. A.; Clauss, M.; Owen-Smith, N., 2012: Black rhinoceros (*Diceros bicornis*) natural diets: comparing iron levels across seasons and geographical locations. *Journal of Zoo and Wildlife Medicine* **43**, S48–S54.

Hummel, J.; Südekum, K.-H.; Streich, W. J.; Clauss, M., 2006: Forage fermentation patterns and their implications for herbivore ingesta retention times. *Functional Ecology* **20**, 989–1002.

Kemperman, R. A.; Bolca, S.; Roger, L. C.; Vaughan, E. E., 2010: Novel approaches for analysing gut microbes and dietary polyphenols: challenges and opportunities. *Microbiology* **156**, 3224–3231.

Kipchumba, A. J., 2002: *Applying foraging theory to diet choice and habitat selection in black rhinoceros*. Doctoral Dissertation. University of Illinois, Chicago.

Ky, I.; Crozier, A.; Cros, G.; Teissedre, P. L., 2014: Polyphenols composition of wine and grape sub-products and potential effects on chronic diseases. *Nutrition and Aging* **2**, 165–177.

Lattimer, J. M.; Cooper, S. R.; Freeman, D. W.; Lalman, D. L., 2007: Effect of yeast culture on in vitro fermentation of a high-concentrate or high-fiber diet using equine fecal inoculum in a Daisy II incubator. *Journal of Animal Science* **85**, 2484–2491.

Lavin, S. R.; Chen, Z.; Abrams, S. A., 2010: Effect of tannic acid on iron absorption in straw-colored fruit bats (*Eidolon helvum*). *Zoo Biology* **29**, 335–343.

Ley, R. E.; Lozupone, C. A.; Hamady, M.; Knight, R.; Gordon, J. I., 2008: Worlds within worlds: evolution of the vertebrate gut microbiota. *Nature Reviews. Microbiology* **6**, 776–788.

Lorenz, M. M.; Alkhafadji, L.; Stringano, E.; Nilsson, S.; Mueller-Harvey, I.; Udén, P., 2014: Relationship between condensed tannin structures and their ability to precipitate feed proteins in the rumen. *Journal of the Science of Food and Agriculture* **94**, 963–968.

Lowman, R. S.; Theodorou, M. K.; Hyslop, J. J.; Dhanoa, M. S.; Cuddeford, D., 1999: Evaluation of an in vitro batch culture technique for estimating the in vivo digestibility and digestible energy content of equine feeds using equine faeces as the source of microbial inoculum. *Animal Feed Science and Technology* **80**, 11–27.

Luchini, N. D.; Broderick, G. A.; Combs, D. K., 1996: Preservation of ruminal microorganisms for in vitro determination of ruminal protein degradation. *Journal of Animal Science* **74**, 1134–1143.

Makkar, H. P. S.; Sen, S.; Blümmel, M.; Becker, K., 1998: Effects of fractions containing saponins from *Yucca schidigera*, *Quillaja saponaria*, and *Acacia auriculoformis* on rumen fermentation. *Journal of Agricultural and Food Chemistry* **46**, 4324–4328.

Makkar, H. P. S.; Gamble, G.; Becker, K., 1999: Limitation of the butanol–hydrochloric acid–iron assay for bound condensed tannins. *Food Chemistry* **66**, 129–133.

Martin-Carron, N.; Goni, I., 1998: Prior exposure of cecal microflora to grape pomaces does not inhibit in vitro fermentation of pectin. *Journal of Agricultural and Food Chemistry* **46**, 1064–1070.

McDougall, E. I., 1948: Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochemical Journal* **43**, 99–109.

Meng, Q.; Kerley, M. S.; Ludden, P. A.; Belyea, R. L., 1999: Fermentation substrate and dilution rate interact to affect microbial growth and efficiency. *Journal of Animal Science* **77**, 206–214.

Meng, Q. X.; Xia, Z. G.; Kerley, M. S., 2000: The requirement of ruminal degradable protein for non-structural carbohydrate-fermenting microbes and its reaction with dilution rate in continuous culture. *Asian-Australasian Journal of Animal Science* **13**, 1399–1406.

Miller, R. E., 2003: *Rhinoceridae* (Rhinoceroses). In: M. E. Fowler, R. E. Miller (eds), *Zoo and Wild Animal Medicine, Current Therapy*. 5th edn. Elsevier Science, St. Louis, pp. 558–569.

Min, B. R.; Pinchak, W. E.; Anderson, R. C.; Hume, M. E., 2006: In vitro bacterial growth and in vivo ruminal microbiota populations associated with bloat in steers grazing wheat forage. *Journal of Animal Science* **84**, 2873–2882.

Mladěnka, P.; Macáková, K.; Filipský, T.; Zatloukalová, L.; Jahodář, L.; Bovicelli, P.; Silvestri, I. P.; Hrdina, R.; Saso, L., 2011: In vitro analysis of iron chelating activity of flavonoids. *Journal of Inorganic Biochemistry* **105**, 693–701.

Molenaar, F. M.; Sainsbury, A. W.; Waters, M.; Amin, R., 2008: High serum concentrations of iron, transferrin saturation and gamma glutamyl transferase in captive black rhinoceroses (*Diceros bicornis*). *Veterinary Record* **162**, 716–721.

Monsma, D. J.; Marlett, J. A., 1995: Rat cecal inocula produce different patterns of short-chain fatty acids than fecal inocula in in vitro fermentations. *Journal of Nutrition* **125**, 2463–2470.

Monsma, D. J.; Marlett, J. A., 1996: Fermentation of carbohydrate in rat ileal excreta is enhanced with cecal inocula compared with fecal inocula. *Journal of Nutrition* **126**, 554–563.

Naumann, H. D.; Tedeschi, L. O.; Muir, J. P.; Lambert, B. D.; Kothmann, M. M., 2013a: Effect of molecular weight of condensed tannins from warm-season perennial legumes on ruminal methane production in vitro. *Biochemical Systematics and Ecology* **50**, 154–162.

Naumann, H. D.; Muir, J. P.; Lambert, B. D.; Tedeschi, L. O.; Kothmann, M. M., 2013b: Condensed tannins in the ruminant environment: a perspective on biological activity. *Journal of Agricultural Science* **1**, 8–20.

Naumann, H. D.; Hagerman, A. E.; Lambert, B. D.; Muir, J. P.; Tedeschi, L. O.; Kothmann, M. M., 2014: Molecular weight and protein-precipitating ability of condensed tannins from warm-season perennial legumes. *Journal of Plant Interactions* **9**, 212–219.

Oftedal, O. T.; Baer, D. J.; Allen, M. E., 1996: The feeding and nutrition of herbivores. In: D. G. Kleiman, M. E. Allen, K. V. Thompson, S. Lumpkin, H. Harris (eds), *Wild Mammals in Captivity*. The University of Chicago Press, Chicago, pp. 129–137.

Pagán, S.; Wolfe, R. M.; Terrill, T. H.; Muir, J. P., 2009: Effect of drying method and assay methodology on detergent fiber analysis in plants containing condensed tannins. *Animal Feed Science and Technology* **154**, 119–124.

Paglia, D. E.; Tsu, I. H., 2012: Review of laboratory and necropsy evidence for iron storage disease acquired by browser rhinoceroses. *Journal of Zoo and Wildlife Medicine* **43**, S92–S104.

Patra, A. K.; Saxena, J., 2011: Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *Journal of the Science of Food and Agriculture* **91**, 24–37.

Pérez-Jiménez, J.; Arranz, S.; Saura-Calixto, F., 2009: Proanthocyanidin content in foods is largely underestimated in the literature data: an approach to quantification of the missing proanthocyanidins. *Food Research International* **42**, 1381–1388.

Rautio, P.; Bergvall, U. A.; Karonen, M.; Salminen, J. P., 2007: Bitter problems in ecological feeding experiments: commercial tannin preparations and common methods for tannin quantifications. *Biochemical Systematics and Ecology* **35**, 257–262.

Santos, A. S.; Rodrigues, M. A. M.; Bessa, R. J. B.; Ferreira, L. M.; Martin-Rosset, W., 2011: Understanding the equine cecum-colon ecosystem: current knowledge and future perspectives. *Animal* **5**, 48–56.

Scho, P.; Mbugua, D. M.; Pell, A. N., 2001: Analysis of condensed tannins: a review. *Animal Feed Science and Technology* **91**, 21–40.

Steuer, P.; Clauss, M.; Südekum, K.-H.; Hatt, J.-M.; Silinski, S.; Klomburg, S.; Zimmermann, W.; Fickel, J.; Streich, W. J.; Hummel, J., 2010: Comparative investigations on digestion in grazing (*Ceratotherium simum*) and browsing (*Diceros bicornis*) rhinoceroses. *Comparative Biochemistry and Physiology A* **156**, 380–388.

Stevens, C. E.; Hume, I. D., 2004: *Comparative Physiology of the Vertebrate Digestive System*, 2nd edn. Cambridge University Press, Cambridge, UK.

Strumeyer, D. H.; Malin, M. J., 1975: Condensed tannins in grain sorghum: isolation, fractionation, and characterization. *Journal of Agricultural and Food Chemistry* **23**, 909–914.

Sunvold, G. D.; Hussein, H. S.; Fahey, G. C.; Merchen, N. R.; Reinhart, G. A., 1995: In vitro fermentation of cellulose, beet pulp, and citrus pectin using fecal inoculum from cats, dogs, horse, humans, and pigs and ruminal fluid from cattle. *Journal of Animal Science* **73**, 3639–3648.

Tebib, K.; Besançon, P.; Rouanet, J. M., 1996: Effects of dietary grape seed tannin on rat cecal fermentation and colonic bacterial enzymes. *Nutrition Research* **16**, 105–110.

Terrill, T.; Windham, W.; Evans, J.; Hoveland, C., 1994: Effect of drying method and condensed tannin on detergent fiber analysis of sericea lespedeza. *Journal of the Science of Food and Agriculture* **66**, 337–343.

Terrill, T. H.; Wolfe, R. M.; Muir, J. P., 2010: Factors affecting ANKOM^{TM} fiber analysis of forage and browse varying in condensed tannin concentration. *Journal of the Science of Food and Agriculture* **90**, 2723–2726.

Van Soest, P. J. J.; Robertson, J. B. B.; Lewis, B. A. A., 1991: Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* **74**, 3583–3597.

Viveros, A.; Chamorro, S.; Pizarro, M.; Arija, I.; Centeno, C.; Brenes, A., 2011: Effects of dietary polyphenol-rich grape products on intestinal microflora and

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gut morphology in broiler chicks. *Poultry Science* **90**, 566–578.

Ward, A. M.; Hunt, A. S., 2001: Summary of mineral and iron binding polyphenolic plant compound levels in diets offered captive black rhinoceros (*Diceros bicornis*) in 3 zoos and 1 ranch in Texas. In: M. S. Edwards, K. J. Lisi, M. L. Schlegel, R. E. Bray (eds), *Proceedings of the Fourth Conference on Zoo and Wildlife Nutrition*. AZA Nutrition Advisory Group , Lake Buena Vista, FL, pp. 175–188.

Ward, A. M.; Coslik, A. H.; Slifka, K.; Meehan, T.; Griffin, M.; Maslanka, M. T., 2005: Dietary iron absorption and the role of tannins in Eastern (*Diceros bicornis michaeli*) and Southern black rhinoceros (*Diceros bicornis minor*). Nutrition Advisory Group Conference on Zoo and Wildlife Nutrition, pp. 91–92.

Wright, J. B., 1997: Essential fatty acids, total lipid, and condensed tannin in the diet of captive black rhinoceroses (Diceros bicornis) in North America and in browses native to Zimbabwe, Africa. Thesis. Cornell University, Ithica, NY.

Zinn, R. A.; Owens, F. N., 1986: A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. *Canadian Journal of Animal Science* **66**, 157–166.