

CHARACTERIZATION OF GRAPE CONDENSED TANNINS AND THEIR EFFECT
ON BLACK RHINOCEROS (*DICEROS BICORNIS*) HINDGUT FERMENTATION

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CHARACTERIZATION OF GRAPE CONDENSED TANNINS AND THEIR EFFECT
ON BLACK RHINOCEROS (*DICEROS BICORNIS*) HINDGUT FERMENTATION

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A candidate for the degree of Master of Science

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NOMENCLATURE

ADF	Acid Detergent Fiber
BSA	Bovine Serum Albumin
CE	(+)-Catechin Equivalents
CP	Crude Protein
CT	Condensed Tannin
DM	Dry Matter
DMF	Dimethylformamide
FAS	Ferric Ammonium Sulfate
GP	Grape Pomace
GSE	Grape Seed Extract
h	Hour
HPLC	High-Performance Liquid Chromatography
HT	Hydrolyzable Tannin
IOD	Iron Overload Disorder
min	Minute
MOEFF	Microbial Efficiency
NDF	Neutral Detergent Fiber
NH ₃	Ammonia
NH ₃ -N	Ammonia Nitrogen
OM	Organic Matter
OM _m	Microbial Organic Matter

PA	Proanthocyanidin
PPP	Protein Precipitable Phenolics
PRP	Proline-Rich Protein
SDS	Sodium Dodecyl Sulfate
SS	Sodium Sulfite
TAE	Tannic Acid Equivalents
TEA	Triethanolamine
VFA	Volatile Fatty Acid
vol	Volume
wt	Weight

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ABSTRACT

Excessive iron accumulation is observed in greater than 75% of tested captive black rhinoceros (*Diceros bicornis*) and iron overload disorder (**IOD**) is associated with high morbidity and mortality. 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. Black rhinoceros are browsing herbivores and naturally consume a diet with high iron chelator concentrations, such as tannins and other polyphenolics, which considerably decrease iron bioavailability. Grape industry by-products are economical, concentrated condensed tannins sources. The objectives of the first experiment were to evaluate grape pomace (**GP**) and grape seed extract (**GSE**) for potential application in black rhinoceros diets as iron chelators and to characterize tannin composition. Grape seed extract was found to be about 64% condensed tannins (**CT**) and an effective iron chelator. Grape pomace is a more economical, yet variable, tannin source and rosé wine grape pomace was found to contain about 10% CT on a dry matter (**DM**) basis.

Variable effects of polyphenolic compounds on microbial fermentation have been reported, and results appear to depend on type and concentration. This variation makes it imperative to determine potential tannin supplement effects on black rhinoceros hindgut fermentation. Equine *in vivo* studies are 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 objectives of the second experiment were to compare fermentation characteristics and nutrient digestibility between the black rhinoceros and domestic horse and to examine GSE effects on fermentation parameters using a continuous single-flow *in vitro* culture system. Two replicated continuous culture experiments were conducted using domestic horse and black rhinoceros feces as inoculum sources comparing four diets with increasing GSE inclusion (0-4% of DM). Increasing GSE inclusion stimulated microbial growth and fermentation. Domestic horse and black rhinoceros hindgut microflora nutrient digestibility and fermentation responses to GSE did not differ. Contrary to our hypothesis, 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% of DM is unlikely to adversely affect nutrient digestibility or microbial viability and fermentation.

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Resource declines and human actions threaten the viability and survivability of many wild animal populations throughout the world. More than 100,000 black rhinoceros were roaming Africa in the 1960s but population numbers have declined more than 95%, mainly as a result of poaching, leading to classification as Critically Endangered by the International Union for the Conservation of Nature (IUCN red list, 2012). Although numbers of African black rhinoceros have increased, the population is still 90% smaller than three generations ago. While conservationists are working *in situ* to increase enforcement and regulation of anti-poaching laws, about 115 individual black rhinos are managed in captivity in the United States (AZA, 2012) to ensure the health and genetic diversity of an *ex situ* population. Unfortunately, the captive population suffers its own threats. Black rhinoceros in captivity experience diseases not described or observed in wild populations (Dennis et al., 2007) including a high prevalence of iron overload disorder (**IOD**). Excessive iron accumulation is observed in greater than 75% of tested captive black rhinoceros and is associated with high morbidity and mortality (Paglia and Tsu, 2012). 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.

Iron overload disorder is thought to be due to disparity between low iron concentrations and bioavailability in natural diets compared to feed items available in captivity. Black rhinoceros are browsing herbivores and naturally consume a diet with high concentrations of iron chelators, such as tannins and other polyphenolics, which considerably decrease iron bioavailability. Current experimental evidence of the benefits of incorporating polyphenols into diets of captive exotic animals is lacking. Commercially available sources have been tested (Clauss, et al., 2007a; Lavin et al., 2010) but alternative sources must be considered due to prohibitive cost and ecological considerations. By-products of grape production industries, such as grape pomace and grape seed extract, are economical sources of concentrated polyphenolic compounds (Ky et al., 2014) and are evaluated in this research for potential application in black rhinoceros diets as iron chelators.

Feeding exotic animals in captivity requires the application of comparative nutrition. Domestic animal models are frequently used to extrapolate nutrient requirements for rare and endangered species and to evaluate the safety and effectiveness of potential medications and dietary ingredients or supplements. Phylogenetic and morphologically related species provide a basis for comparison. However, species-specific differences are becoming apparent and limitations of some domestic animal models have been identified. Therefore, assessing the domestic horse as an appropriate model for nutrient digestion and absorption in black rhinoceros is valuable.

This review will discuss IOD etiology and potential solutions, focusing on supplementation of tannins as iron chelators. Iron overload disorder is thought to be due

to disparity between low iron concentrations and bioavailability in natural diets compared to feed items available in captivity. Black rhinoceros are browsing herbivores and naturally consume a diet with high concentrations of iron chelators, such as tannins and other polyphenolics, which considerably decrease iron bioavailability.

IRON OVERLOAD DISORDER

Iron overload in captive wild animal species

Iron overload disorder is characterized by excessive iron accumulation in body tissues and occurs in a variety of captive wild animal species, as well as in humans. Iron overload disorder is often associated with pathologic changes and increased susceptibility to disease. Other terms related to IOD include hemosiderosis, focal iron deposition without associated tissue damage, and hemochromatosis, excessive iron accumulation resulting in tissue damage. Although IOD is observed across many species, the etiology and manifestations of the disorder are quite varied.

Species prone to IOD generally fit into one of the following ecological classifications: browsing herbivores, frugivores, or insectivores (Clauss and Paglia, 2012), however, exceptions do exist. Hemosiderosis and hemochromatosis have been described in numerous species. Clauss and Paglia (2012) provide an excellent review on the breadth and diversity of species in which IOD has been described. Browsing Perissodactyl species such as the African black and Sumatran rhinoceroses (Candra et al., 2012; Olias et al., 2012) and tapirs (Bonar et al., 2006)) are commonly afflicted with excessive body iron stores. Human hemochromatosis is a hereditary disorder associated

with a mutation in a gene that codes an important iron regulatory protein, whereas, the most commonly proposed etiology of IOD in browsing rhinoceros is the difference between iron concentration and bioavailability in wild versus captive diets.

Iron overload in browsing rhinoceros species

As strict browsing species, the black and Sumatran rhinoceros (*Dicerorhinus sumatrensis*) did not evolve mechanisms to protect them against iron overload because their natural diet contains lower levels of available iron than diets fed in captivity (Smith et al., 1995; Candra et al., 2012; Ganz and Nemeth, 2012). Studies examining this disparity reported an average of 91 ppm iron in wild diets whereas zoo diets averaged 374 ppm (Helary et al., 2012). Deficiencies in dietary fiber and low to nonexistent concentrations of natural iron chelators, such as polyphenols and phytate, exacerbate this problem. Beutler et al. (2001) explored the possibility of genetic differences in iron regulatory gene regions between black rhinoceros and other grazing rhinoceros species, which do not accumulate pathogenic iron loads. A slightly different polymorphism was identified in the HFE gene region of black rhinoceros, but not Sumatran, and is not thought to result in any functional differences. Another hypothesis is a species-specific difference in iron metabolism. Numerous studies suggest that black rhinos have innate differences in red blood cell metabolism and immunologic capabilities (Dierenfeld et al., 2005; Miller et al., 2012). Despite the unknown molecular mechanism, leading researchers agree IOD in captive browsing rhinoceros species is clearly the result of an

evolutionary adaptation to low iron diets in combination with high dietary iron concentrations in captivity (Klopfleisch and Olias, 2012).

At necropsy examinations, IOD is generally not associated with gross lesions but must be diagnosed through microscopic examination of tissues (Klopfleisch and Olias, 2012; Olias et al., 2012). Manifestations of iron overload include hemosiderin laden macrophages (especially liver Kupffer cells) and widespread iron deposition in the spleen, liver, bone, small intestine villi, and lungs (Paglia and Radcliffe, 2000; Citino et al., 2012; Paglia and Tsu, 2012). Hemosiderosis was investigated but not reported in free-ranging black rhinos. Test subjects were found to have iron analyte values comparable to normal equine and human control values, as well as those of captive grazing rhinoceros (*Ceratotherium simum* and *Rhinoceros unicornis*) (Paglia and Radcliffe, 2000). In comparison, serum ferritin levels and hepatic nonheme iron concentrations in captive black rhinoceros increase logarithmically with age and time in captivity (Smith et al., 1995; Dennis et al., 2007; Paglia and Tsu, 2012).

Kock et al. (1992) and Smith et al. (1995) first identified excessive iron storage as a predisposing factor for a number of secondary diseases with high morbidity and mortality. Black rhinoceros are inherently sensitive to oxidative stress (Paglia and Miller, 1993) and have naturally low levels of biological antioxidants (Dierenfeld, 1989). Increasing iron loads further disrupts normal physiological functions, increases susceptibility to infection, and is associated with hemolytic anemia, mucocutaneous ulcerative disorder, and stress intolerance (Khan et al., 2007; Olias et al., 2012).

Iron overload solutions and prevention strategies

IOD is an important and pressing issue in zoo nutrition. Nutritionists initially addressed this problem by removing supplemental iron sources and food items especially high in iron or nutrients that increase iron bioavailability (ex. citric acid) (Clauss et al., 2012). Diets fed to browsing rhinoceros often contain excessive iron levels for many reasons. Hay iron content varies as a result of growing conditions, use of fertilizers, or soil contamination in the forages (legume and grass forage ranged from 10-2,599 ppm Fe (Adams, 1975)). Pelleted feeds are a significant source of iron due to common ingredients such as beet pulp (85-100 ppm), soybean meal (110-240 ppm), and dicalcium phosphate (20-11,100 ppm). The nutrition working group for the International Workshop on Iron Overload Disorder in Browsing Rhinoceros (February 2011) recommended providing only 50-100 ppm iron for these species and browse should be offered as often as possible. Practical limitations and limited availability of necessary dietary components (especially browse) make this goal difficult to achieve. Commercial diets have been formulated to contain minimal iron, however, concentrations continue to be greater than those recommended for iron sensitive species (Mylniczenko et al., 2012). Other interventions such as therapeutic phlebotomy have been used successfully to decrease body iron loads in humans and other species (Venn-Watson et al., 2012), but phlebotomy is not a widely attempted treatment in rhinos as it involves serial immobilization, specialized facilities, and intensive training management (Mylniczenko et al., 2012). Ultimately, prevention of excessive iron absorption is necessary to prevent the onset of this disease.

TANNINS IN ANIMAL NUTRITION

The International Workshop on Iron Overload Disorder (2011) prioritized and encouraged research to identify, characterize, and make recommendations on the use of compounds that sequester dietary iron. Polyphenolic compounds are well documented iron chelators and are found in substantial concentrations in natural diets of many wild animals, including browsing rhinoceros, in comparison to most diets fed in zoological institutions (Clauss, 2003). This discrepancy and the high incidence of related nutritional diseases reported in iron sensitive captive species, confirms the importance of researching tannin application in captive diets

Tannin sources and animal interactions

Plants produce polyphenols as secondary metabolites. They are involved in diverse processes such as growth, lignification, pigmentation, pollination and resistance against pathogens, predators and environmental stresses (Haslam, 1998; Fraga et al., 2010). These compounds contribute to the color, structure, chemical properties, nutritive value and palatability of plant products (Mehansho et al., 1987). Tannins are a classification of polyphenols and are generally described as water-soluble compounds with molecular weights greater than 500 Daltons with the ability to precipitate protein (Hagerman and Butler, 1981). Most importantly, polyphenols are well documented iron chelators (Khokhar and Owusu Apenten, 2003; Gaffney et al., 2004; Lavin, 2012).

Animal-polyphenol interaction is dependent upon the chemical diversity of polyphenolic compounds found in a huge variety of food items. More than 8,000 polyphenolic compounds have been identified in foods including fruits, vegetables, tea, wine, coffee, chocolate, some cereal grains, and olive oil (Zern and Fernandez, 2005; Perron and Brumaghim, 2009). Manach et al. (2004) provides a review of common food sources and bioavailability of polyphenols in human diets. A database providing food polyphenol composition can be used to estimate intake of individual compounds or groups of compounds based on dietary records (<http://www.phenol-explorer.eu>) and search published literature on polyphenol containing foods and associated metabolites.

Browse is a common source of polyphenols in wild herbivore diets. These compounds are contained within the vacuoles of plants and are found in buds, leaves, roots, seeds, and stems. Tannins are widely distributed in the plant kingdom and are common both in gymnosperms and angiosperms. Within angiosperms, tannins are more commonly found in dicotyledons than in monocotyledons. In natural browsing rhinoceros diets, tannin concentrations average between 2-5% of diet dry matter depending on season and geographical location (Helary et al., 2012).

Further complicating the animal-tannin relationship are numerous equivocal reports on the ecological purpose of and biological responses to tannins. Some support the hypothesis that tannins act as antiherbivory defense by binding with dietary protein and digestive enzymes (Robbins et al., 1987), whereas other studies suggest that toxicity, rather than digestion inhibition, is the mode of action (Clausen et al., 1990). There are also reports that tannins have no detectable effect on food intake (Clauss et al., 2007a;

Davies et al., 2009). Clausen et al. (1990) demonstrated that when snowshoe hares (*Lepus americanus*) were offered equal amounts of condensed tannins from two different plant species, they rejected one over the other and contrary to the authors' hypothesis actually preferred the plant with greater protein precipitation ability. African black rhinoceros populations have been observed consuming individual plants with condensed tannin concentrations greater than 10%, but these are generally avoided if lower-tannin options are available (Kipchumba, 2002). Many studies have noted large inter-species and even inter-individual variation in acceptance of tannin-containing food items (Ayres et al., 1997; Al-Mamary et al., 2001; Clauss et al., 2003; Davies et al., 2009; Wren et al., 2013).

Tannin chemistry

While it is of interest to review specific reports of biological and behavioral effects of tannin intake, it is important to remember the term “tannin”, or even more specific terms such as “condensed tannins”, cannot be universally applied and compared due to the chemical complexity of these large polyphenolic classes. Tannins are highly polymerized polyphenolic compounds that are generally divided into two classes, hydrolyzable and condensed tannins. Hydrolyzable tannins (**HT**; Figure 1) are esters of a non-phenolic sugar, usually D-glucose, and gallic acid or its derivative (Hagerman, 1992). These compounds hydrolyze easily in acidic or basic conditions to yield the parent polyol and the phenolic acids.

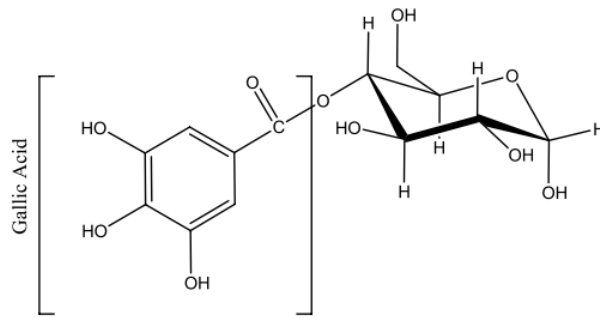


Figure 1-1. Structure of a hydrolyzable tannin; gallic acid esterified to glucose.

Condensed tannins (CT) are chemically defined as polymerized flavan-3-ols, a subclass of flavonoids (Fraga, 2007), with a basic structure (Figure 2) based on a common C6-C3-C6 carbon skeleton. Flavan-3-ols are distinct from other flavonoid classes commonly found in plants because they are present as monomer units (ex. (-)-epicatechin and (+)-catechin, depicted in Figure 3.), gallate derivatives of the monomers (ex. (-)-epigallocatechin gallate, the most abundant tea polyphenol), or as oligomers (i.e. proanthocyanidins or condensed tannins, Figure 4.) (Crozier et al., 2006). Condensed tannins do not undergo hydrolysis in acid or base, but in hot alcohol the flavonoid polymer is oxidatively cleaved to yield colored anthocyanidins, thus these compounds are often called “proanthocyanidins” (Hagerman et al., 1992). This reaction is the basis for the most common analytical method for CT, the acid butanol method (Hagerman, 2002).

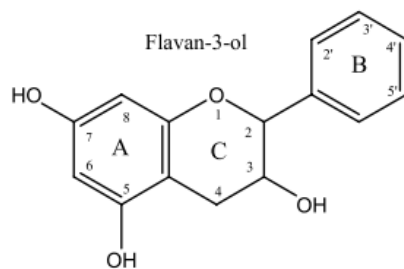


Figure 1-2. Basic structure of flavan-3-ols indicating the rings (A, C and B) and substitution position numbers

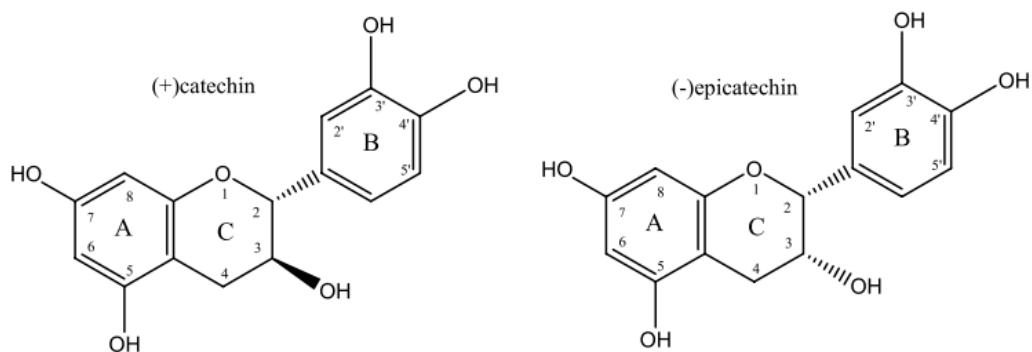


Figure 1-3. Structures of common flavan-3-ol monomers, catechin and epicatechin

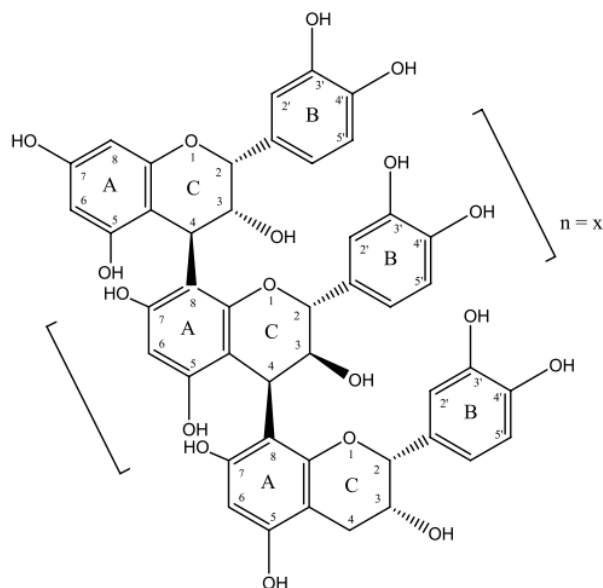


Figure 1-4. Basic condensed tannin (proanthocyanidins) structure

Condensed tannins are characterized based on hydroxylation patterns, stereochemistry, functional groups, interflavan linkages, and degree of polymerization (Ayres et al., 1997; Naumann et al., 2013a). Each of these chemical characteristics relates some specific functionality to the compounds. A flavan-3-ol unit with a tri-hydroxylated B ring is termed a prodelphinidin and a di-hydroxylated B ring is called a procyanidin (ex. the monomer units of the CT in figure 4.) (Foo and Porter, 1980). The procyanidin:prodelphinidin ratio of plant CT, analyzed by HPLC, 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). The type of interflavan linkage may be related to solubility in water (Bate-Smith, 1975) and degree of polymerization into dimers, trimers, tetramers, and higher oligomers can affect its protein precipitation ability (Harbertson et al., 2014; Lorenz et al., 2014). Galloylation at the C3 position increases

the potential for hydrogen bonding (Harbertson et al., 2014) and may be related to iron chelation capacity (Andjelkovic et al., 2006).

Biological activity

Polyphenolics exert a wide range of biological effects resulting in numerous potential health benefits and economical responses in animal agriculture. Well-documented effects of these diverse compounds range from potent antioxidant and cryoprotective activity to increased N utilization and decreased methane production in ruminants. The ability of tannins to bind protein and chelate metal ions are the basic chemical mechanisms underlying many important biological activities.

Protein binding

Chemical mechanisms

One characterizing factor of tannins is the ability to precipitate protein out of a solution (Hagerman and Butler, 1978) and has been the subject of the majority of tannin research as it relates to biological activity, ecological relationships with herbivores, or taste perception in wine. Tannin-protein interaction research to date strongly indicates that precipitation increases with increasing tannin size (Porter and Woodruffe, 1984) and that the mode of interaction is a combination of hydrogen bonding and hydrophobic interactions (Hagerman et al., 1998; Charlton et al., 2002). However, several aspects about tannin-protein interaction are still not well understood (Harbertson et al., 2014). The reaction is not site specific, but involves multivalent cross-linking, stabilized by

hydrogen bonding between the phenolic hydroxyl groups and the amide carbonyl of the peptide backbone (Hagerman, 1992). Interactions between these two compounds are selective with tannins preferentially binding to proteins with more open structure such as proline rich proteins rather than other globular proteins (Hagerman and Butler, 1981; Li, 2014). High proline content promotes hydrogen bonding by both imposing secondary structures that expose the peptide backbone and by strong hydrogen bonding through the tertiary amide bond (Hagerman, 1992; Hagerman, 2012).

Precipitation depends on the chemical structure of both protein and tannin, protein charge and isoelectric point, degree of tannin polymerization, relative concentrations of tannin and protein, pH and ionic strength and temperature of the solution (Hagerman et al., 1998; Adamczyk et al., 2012). Condensed tannin-protein binding affinity is greatest when the pH is near the isoelectric point of the protein (Hagerman and Butler, 1981) and complexes dissociate at pH conditions of 3 or less. Small polyphenols do not normally precipitate protein because they lack multiple phenolic sites required for multivalent binding and cross-linking (Hagerman, 2012).

Influence on animal nutrition

Condensed tannin supplementation decreased protein digestibility in some studies (Robbins et al., 1991; Hagerman et al., 1992; Spalinger et al., 2010), whereas in others no effect on true protein availability was observed (Clauss et al., 2007a; Davies et al., 2009). A decrease in protein absorption is attributed to the strong protein precipitating ability of tannins, but may be a biased effect due to increased endogenous or microbial protein

excretion (Shahkhalili et al., 1990; Getachew et al., 2000; Al-Dobaib, 2009). Tannins are released from the plant matrix during mastication and become available to bind with surrounding nutrients. Protein is preferentially bound whether that is dietary protein, salivary proline-rich proteins (**PRP**), or microbial protein. Some mammalian species, including black rhinoceros (Clauss et al., 2005b) are thought to secrete tannin-binding salivary proteins as a defense mechanism against tannins (Robbins et al., 1991; Shimada, 2006). While many tannin-protein complexes dissociate in the acidic stomach, the salivary PRP-polyphenol complex is robust and stable through digestion (Mehansho et al., 1987; Hagerman, 2012) and may decrease negative effects on protein digestion when herbivores consume high tannin diets.

Tannins bind dietary protein with varying affinity due to previously discussed characteristics of both tannins and proteins. In ruminants, CT and protein bind in the neutral pH conditions of the rumen (McNabb et al., 1996) thus, decreasing protein solubilization and ruminal degradation rate (McNabb et al., 1996; Min et al., 2005; Bruno-Soares et al., 2011). Additionally, CTs have been shown to decrease microbial proteolytic enzyme activity (Patra and Saxena, 2011). Greater fractions of ruminally undegradable protein increases the flow of dietary amino acids to the abomasum and small intestine which has been shown to improve nitrogen utilization (Waghorn et al., 1994; Patra and Saxena, 2011) and cattle growth and feed efficiency (Lehmkuhler and Kerley, 2007; Hersom et al., 2009). For the same reasons, ruminal ammonia nitrogen concentrations may decrease due to tannin supplementation (Puchala et al., 2005; Alipour and Rouzbehan, 2010).

In both ruminant and non-ruminant animals most CT-protein complexes, depending on the binding affinity, dissociate in the acidic stomach releasing both compounds into the digestion matrix (Patra and Saxena, 2011). Neutral pH conditions in the small intestine provide another opportunity for tannin-nutrient binding, although complexes are less likely to form at a pH > 7 (Patra and Saxena, 2011). Affinity and binding strength of tannin-protein interactions affects protein digestibility throughout the digestive tract. Kariuki and Norton (2008) measured greater than 82% true digestibility of bovine serum albumin protein in sheep introduced through an abomasum cannula as a complex with CT, suggesting the majority of CT bound protein was released and available post ruminally. Clauss et al. (2007a) supplemented black rhinoceros with either quebracho tannin (source of CT) or tannic acid (hydrolyzable tannin) up to 1.5% of diet DM and measured no influence of tannin supplementation on crude protein digestibility. Tannins can bind other endogenous proteins in the intestinal tract, such as digestive enzymes (Bravo, 1998) and inhibit their activity (He et al., 2007). This may cause further reduction in the protein digestibility (Al-Mamary et al., 2001) as well as other macronutrients, such as starch and lipids (Mcdougall and Stewart, 2005). Interactions with microbial enzymes are discussed in a later section of this review.

Mineral chelation

Metal ion complexation is one of the mechanisms through which tannins act as antioxidants. In biological systems, redox active metals catalyze free radical-producing reactions. Thus, sequestration of iron by tannins prevents metal-catalyzed free radical

formation through Fenton-type reactions. (Khokhar and Owusu Apenten, 2003; Perron and Brumaghim, 2009). Additionally, stable tannin chelation of dietary iron has been effective at reducing iron absorption in captive wild animal species sensitive to iron overload (Seibels et al., 2003; Lavin et al., 2010).

Chemical mechanisms

Early studies investigating iron absorption inhibition by phenolic compounds indicated a close relationship between the amount of TA added to a meal and the degree of inhibition (Brune et al., 1989). Since then, functional groups important for iron and other mineral binding have been identified. Tannins with catechol and gallol groups are effective metal chelators (Andjelkovic et al., 2006; Perron and Brumaghim, 2009). However, large differences have been observed in the metal-chelating capacity of different polyphenols (Perron and Brumaghim, 2009; Fraga et al., 2010) and from these studies there is consensus on the presence of iron binding sites. A 3',4'-dihydroxygroup on a flavonoid B ring (Figure 5) is required for iron binding (Khokhar and Owusu Apenten, 2003) and increased free hydroxyl groups are associated with increased iron binding ability (Andjelkovic et al., 2006; Mladěnka et al., 2011).

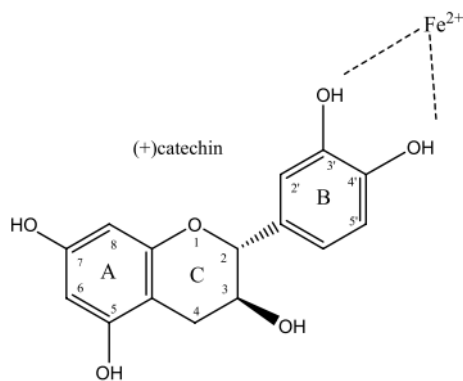


Figure 1-5. Catechin, a condensed tannin monomer, binding to ferrous iron, in the 3',4'-dihydroxy position of the B ring

Tannins influence on mineral bioavailability

Tannins and other polyphenols efficiently bind iron (both Fe^{3+} and Fe^{2+}) and to a lesser extent, copper, manganese, aluminum, and zinc. However, the extent to which polyphenol-metal chelation affects bioavailability of the mineral varies greatly (Greger and Lyle, 1988; Afsana et al., 2004). Iron-tannin complexes are generally very stable throughout the gastrointestinal tract and can effectively inhibit absorption (Hurrell and Cook, 1999; Glahn and Wortley, 2002; Seibels et al., 2003; Lavin et al., 2010; Wren et al., 2013) at levels as low as 5 g AT/kg (Afsana et al., 2004). In cell culture, a 1:1 ratio of TA to Fe inhibited 92% of iron absorption (Glahn and Wortley, 2002). When applied in a more complicated food matrix as part of a meal this effect was lessened but still evident (Yun et al., 2004), suggesting that degree of mineral absorption inhibition is influenced by the presence of other nutrients in the food matrix during digestion.

Zinc has a much lower affinity for polyphenols than iron, particularly at acidic and neutral pH conditions (Santos-Buelga and Scalbert, 2000). *In vivo* studies have

demonstrated no effect of tannin supplementation on zinc absorption (Afsana et al., 2004). Tea consumption has been reported to inhibit aluminum absorption in humans and rats (Fairweather-Tait et al., 1991) but was found to have no effect in other studies (Greger and Lyle, 1988).

The strong metal chelation ability of polyphenol sources makes it imperative to screen supplements considered for dietary iron chelation in animal diets for inherent metal content. Often these sources will pick up metals either from the growing environment or through processing and may actually contribute more metals to the diet than they can sequester (Hagerman, personal communication, October 6, 2014). Additionally, polyphenols that are already bound to minerals or proteins are unavailable to bind with other minerals and may result in ineffective supplementation.

Bioavailability

Some biological effects of polyphenolics are related to their interaction with nutrients in the gastrointestinal lumen, but many of the promoted health benefits require absorption and bioavailability of the compounds. Condensed tannins are poorly absorbed in the gut due to their polymeric nature and high molecular weight (Manach et al., 2005). It is estimated that more than 90% of ingested CT are not absorbed in the small intestine and pass on to the colon where the compounds are extensively metabolized by gut microflora to produce smaller molecules, including simple phenolic acids (Choy and Waterhouse, 2014). Consequently, biological effects may be due to actions of more readily absorbed metabolites. In contrast, hydrolyzable tannins are easily cleaved in

acidic stomach conditions into the sugar core molecule and phenolic acid derivatives (Reed, 1995) and have been associated with toxicity in a variety of domestic animals (Mueller-Harvey, 2006).

Metabolites have been identified in the blood and urine of humans (Crozier et al., 2010) and rats after oral administration of proanthocyanidin-rich grape seed extract (Tsang et al., 2007; Prasain et al., 2009) and concentrations corresponded to 3-4% of the monomers ingested. The data indicated that oligomeric proanthocyanidins are not depolymerized into monomeric flavan-3-ols during digestion and passage through the stomach and GI tract (Tsang et al., 2007). Prasain et al. (2009) concluded that monomer catechins and proanthocyanidins up to trimers were absorbed from the GI tract and were present in the blood and urine. By feeding rats purified catechin, dimers, trimers or procyanidin polymers, Gonthier et al. (2003) showed that the extent of degradation into aromatic acids decreased as the degree of polymerization increased. Since higher molecular weight CT are not absorbed intact and are highly resistant to mammalian digestive enzymes, it is generally believed that they are absorbed from the colon following microbial fermentation (Gonthier et al., 2003), if at all.

Microbial-tannin interaction and metabolism

Microbial metabolism is a critical factor affecting tannin bioavailability. Gut microflora may depolymerize CT producing more small, bioavailable proanthocyanidins, or may catalyze ring fission reactions (Deprez et al., 2000). Additional functional group cleavage reactions (ex. dehydroxylation, demethylation and decarboxylation) may occur.

Phenolic compounds can be further metabolized by microflora only after deconjugation or deglycosylation has occurred (Aura, 2008). Microbial tannases that hydrolyze galloyl esters are present in the rumen and allow further metabolism to pyrogallol and other low molecular weight phenols that are absorbed from the rumen (Reed, 1995). Bravo et al. (1994) measured low recovery of catechin (3% of ingested) and tannic acid (4.5%) in feces, indicating that these compounds are almost completely digested, absorbed, or modified in the intestinal tracts of rats.

Recent interest in the gut microbiome offers new potential to understand the variability of reported polyphenol bioactivities and selective pressure on gut microbial populations (Aura, 2008; Gross et al., 2010; Kemperman et al., 2010; Dall'Asta et al., 2012; Margalef et al., 2014). Historically, tannins have been regarded as inhibitory to the growth of rumen microbes (Patra and Saxena, 2011) and the antimicrobial properties have been investigated (Jones et al., 1994; Anderson et al., 2012; Ranjitha et al., 2014). Suggested mechanisms of action include: direct binding to bacterial cell membranes, thus disturbing membrane functions and inhibiting cell growth; formation of complexes with metal ions and protein leading to decreased growth substrate availability; enzyme inhibition; and reactive oxygen generation (Smith et al., 2005; Kemperman et al., 2010). Condensed tannins have been reported to inhibit the growth of proteolytic bacteria (Min et al., 2006; Patra and Saxena, 2011) further decreasing ruminal protein fermentation.

Although some bacterial populations are inhibited, others can thrive in the vacant niche of the ecosystem (Kemperman et al., 2010). Viveros et al. (2011) observed greater cecal microbial biodiversity in chickens fed grape pomace concentrate and grape seed

extract. In addition, potential phenol-degrading bacteria and other unidentified organisms were detected. Supplementation with CT, but not monomers, has been associated with increased volatile fatty acid (VFA) concentration in rat cecum at 7% of diet DM (Bravo et al., 1994; Tebib et al., 1996). However, there was no increase in fecal VFAs when fed at 1.5% of dry matter (DM) to black rhinoceros (Clauss et al., 2007a). More research is needed to evaluate general and species-specific effects of different tannin compounds on microbiome composition. Next generation sequencing platforms, microarrays, and high-throughput metaproteomics and metabolomics provide an opportunity to explore the taxonomic and functional diversity in gut microbial populations and examine the influence of these bioactive molecules.

GRAPE TANNINS AS A VIABLE SOLUTION FOR IRON OVERLOAD DISORDER

Black rhinoceros *in vivo* studies

Many researchers suggest that browsing rhinoceros and other species susceptible to IOD should benefit from the addition of tannins to their diet (Paglia et al., 2001; Clauss, 2003; Gaffney et al., 2004; Clauss et al., 2012; Helary et al., 2012; Lavin, 2012). Ideally, browse would be offered as the main constituent of the diet, eliminating the need to provide exogenous tannins or synthetic iron chelators. Unfortunately, this is an unrealistic goal in many geographical regions in which black rhinoceros are managed. Alternatives must be further investigated to find feasible, effective prevention solutions for the nutritional and health stresses this species experiences under human management.

Tannins have been successfully supplemented to reduce iron absorption in other IOD-prone species (Seibels et al., 2003; Lavin et al., 2010) but there has been only one set of studies investigating tannin supplementation to black rhinoceros. Clauss and others (2005b, 2006b, 2007a, and 2007b) conducted feeding trials with eight black rhinos from 3 different zoos. Tannins were added to the animal's regular zoo diets by incorporating 5% tannic acid (hydrolyzable tannin) or 5% quebracho (condensed tannin) in the pellet component, resulting in dietary tannin concentrations of 0.5-1.5% of DM. Tannic acid supplementation increased both the tannic acid-binding and quebracho-binding capacity of black rhinoceroses saliva. Quebracho supplementation did not result in an increase in TA-binding capacity compared to the control diet, but did increase the quebracho-binding capacity of saliva (Clauss, et al., 2005b). This study was the first to indicate that black rhinos may increase their production of salivary tannin-binding protein in response to increased levels of dietary tannin.

Acceptance and digestive coefficients due to tannin supplementation were also measured (Clauss, et al., 2007a). Food intake did not differ between the quebracho diet and control, but increased when the animals were fed tannic acid-containing pellets, suggesting tannin supplementation (either condensed or hydrolyzable) at low concentrations (0.5-1.5% of DM) is unlikely to negatively impact diet acceptance by black rhinoceros. Tannin supplementation did not affect total tract apparent DM or CP digestibility and total short chain fatty acid content of fecal water did not differ due to tannin supplementation. Additionally, there was no difference in apparent iron absorption, or any other mineral, between the tannin supplemented diets and control

(Clauss, et al., 2007b). This study indicates that dietary tannin concentrations of 1.5% were too low to observe an effect on iron availability. Lastly, it was found that quebracho supplementation, but not tannic acid, decreased the number of fecal *Enterobacteriaceae* colony forming units, and increased the total antioxidant capacity of the feces. This suggests that unlike HT, CT were not absorbed or degraded to a significant extent throughout the digestive tract and could potentially reduce the number of potentially pathogenic intestinal bacteria and improve gut antioxidant capacity.

Application of tannin supplementation into zoo diets for IOD prone species will require the use of tannin sources other than tannic acid or quebracho. The use of these commercially available sources is prohibitive due to economical and ecological reasons (Clauss, 2003; Gaffney et al., 2004). Polyphenolics extracted from grapes are potentially a viable solution.

Grape pomace and grape seed extract

Grape skins and seeds are rich sources of flavonoids including monomeric polyphenolic compounds, such as catechins, epicatechins, and epicatechin-3-O-gallate, and dimeric, trimeric, and oligomeric procyanidins (Lu and Foo, 1999; Kammerer et al., 2004; Yilmaz and Toldeo, 2004; Makris et al., 2007; Brenes et al., 2008). By-products from grape production industries (wine and grape juice) account for 30% (wt/wt) of the grapes produced (Makris et al., 2007) and world grape pomace production averages 7-9 million tons per year (Dwyer et al., 2014). This large quantity of waste is an economical loss and an ecological problem (Llobera and Cañellas, 2007; Ping et al., 2011). Grape

pomace includes the grape skins, seeds, and sometimes stems and leaves, and retains about 70% of the polyphenolics found in the original products (Dwyer et al., 2014). Grape seeds can then be removed from pomace and be further extracted to produce grape seed extract (**GSE**), a highly concentrated source of proanthocyanidins (The Grape Seed Method Evaluation Committee, 2001).

A commercial GSE product, ActiVin[®] is prepared by water extraction and has been thoroughly evaluated for safety and toxicity in rats and mice (Bagchi et al., 2000; Wren et al., 2013). Grape seed extract contains approximately 75% oligomeric polyphenols and has been found to decrease levels of serum iron and the serum iron/total iron binding capacity ratio by 14-17% when supplemented at 2% of DM to rats (Wren et al., 2013). Other documented health benefits include anticancer properties (Krohn et al., 1999; Ye et al., 1999) and decreased gastrointestinal oxidative stress (Bagchi et al., 1999). Horses fed GSE (2.2 – 7.3 g/kg DM) displayed no adverse health effects and GSE did not affect feed or water intake (Davies et al., 2009). This commercially available product is marketed for the human nutraceutical industry and may be cost prohibitive for continuous supplementation in black rhinoceros diets. However, when compared to the cost of synthetic iron chelation medication, it may be feasible. Alternatively, grape pomace obtained from local wineries may be a more viable option.

The vinification method applied to the grape variety greatly affects pomace properties and tannin composition (Makris et al., 2007; Dwyer et al., 2014). Lightly pressed varieties of five red wine pomace samples in Missouri were found to have the greatest concentrations of total polyphenols, CT, antioxidant activity and iron-binding

phenolic capacity (Spradling, 2008). Ruberto et al. (2007) also found significant variation in proanthocyanidin concentration and composition between five Sicilian red grape wine cultivars. Furthermore, vintage and ripening status of the grapes may be responsible for inter-batch variation (Kammerer et al., 2004). These studies reinforce the importance of screening potential grape pomace for CT content, iron binding capacity and mineral content before supplementing captive black rhinoceros diets.

DOMESTIC ANIMAL MODELS IN COMPARATIVE NUTRITION

While the nutrient requirements of most wildlife species remain unknown, extrapolation from domestic animal models can be useful. There have been vast advances and improvements in comparative nutrition yet problems still persist in feeding captive wildlife in zoos, possibly from inappropriate application of domestic animal models. Dierenfeld (1996) addressed some of these inadequacies:

“With wildlife and exotics, species-specific differences are becoming apparent, and limitations of domestic animal models are being identified. We recognize that although the basis of nutritional requirements can be found in domestic models, our production goals in zoo populations differ distinctly from those of the pet or livestock industry. Furthermore, the unique metabolisms, behaviors, and physiologies of numerous species are simply not duplicated in domestic animal models. Thus, numerous disciplines encompass the feeding of zoo animals.”

It is important to evaluate the appropriateness of nutrition models used for wild animal species, especially when nutritionally related problems are consistently identified in

captive populations. The high prevalence and severe health consequences of iron overload suggests a need to reevaluate the domestic horse model for browsing rhinoceros nutrition.

Domestic horse model for rhinoceros nutrition

As large hindgut fermenting herbivores, the gastrointestinal anatomy of rhinoceros most resembles that of equids (Clemens and Maloiy, 1982; Stevens and Hume, 2004) and the domestic horse has frequently been used as a nutritional model for all rhinoceros species (Ofstedal et al., 1996; Dierenfeld, 1999; Miller, 2003; Marcus Clauss et al., 2006a). This model has been confirmed for grazing rhinoceros species (African white (*Ceratotherium simum*) and Indian (*Rhinoceros unicornis*) (Frape et al., 1982; Clauss et al., 2005a)) and was found to be adequate for captive tapir nutrition as well (Clauss et al., 2009). However, inadequacies of the horse model have been reported for elephants (Ofstedal et al., 1996).

Mineral composition of natural forages in black rhino diets differs from both the natural diet of grazing equids and from NRC horse recommendations (Dierenfeld, 1999; National Research Council, 2007). Clauss, et al., (2007b) found that black rhinos had significantly greater calcium and magnesium absorption efficiency than domestic horses and greater endogenous sodium loss despite similar absorption efficiency. This suggests that differences in macromineral absorptions exist between the two species. Additionally, incidence of iron overload in horses is much less frequent, making the horse a

questionable model for iron metabolism and IOD in browser rhinos (Clauss and Paglia, 2012).

Fermentation of structural carbohydrates and undigested dietary components occurs in the cecum of these species, with the products of fermentation (VFAs) significantly contributing to overall energy metabolism of the animal. Cecal fermentation has never been compared between browsing rhinoceros and horses but may explain differences observed in digestibility of similar diets between the two species. Fundamental differences in digestive physiology between grazing and browsing species suggests fermentation may differ (Clauss et al., 2005a; Clauss et al., 2006a; Hummel et al., 2006). Variable effects of polyphenols on microbial fermentation (Tebib et al., 1996; Martin-Carron and Goni, 1998) makes it imperative to determine effects of potential supplements on black rhinoceros microbial populations, fermentation characteristics, and overall nutrient digestibility. Equine *in vivo* studies are often used to assess diets or supplements for rhinoceros. Therefore, evaluating the use of a domestic horse model for black rhinoceros fermentation is crucial.

CONCLUSIONS

In summary, iron overload disorder is a prevalent issue in black rhinoceros zoo populations and is associated with high morbidity and mortality. Prevention strategies must limit dietary iron concentration and bioavailability. Tannins are a chemically diverse class of polyphenolics, characterized by protein precipitating and iron binding activity. Tannin chelation of dietary iron has effectively reduced iron absorption in other

IOD-prone captive wild animal species but more research specific to tannin supplementation in rhinoceros diets is necessary. Grape seed extract is a concentrated source of condensed tannins that may have application as an iron chelation supplement in rhinoceros diets. Assessing biological responses of black rhinoceros to dietary tannin supplementation is important. Evaluation of iron absorption *in vitro* models and comparison of responses to tannins between black rhinoceros and their nutritional model, the domestic horse, is valuable.

CHAPTER 2

CHARACTERIZATION OF GRAPE SEED EXTRACT AND GRAPE POMACE CONDENSED TANNINS

ABSTRACT

Diets of many wild animals contain substantial concentrations of polyphenols, which significantly influence nutrient availability, gastrointestinal and systemic health, and gut microbial populations. Condensed tannins (CT), a classification of polyphenolics, are a chemically and functionally diverse group of secondary plant metabolites. The natural diet of black rhinoceros (*Diceros bicornis*) contains between 1-5% CT, which considerably decrease dietary iron bioavailability. Captive black rhinoceros diets are tannin deficient resulting in excessive iron accumulation associated with high morbidity and mortality. The objective of this study was to evaluate grape seed extract (GSE) and grape pomace (GP) tannin functional and chemical characteristics for application in captive black rhinoceros diets to prevent excessive iron absorption. The acid butanol, protein precipitable phenolics (PPP), and iron-binding capacity assays were used to quantify GSE and GP CT concentration. High-performance liquid chromatography was used to characterize CT chemical composition. Grape seed extract was found to be a more effective source of CT for supplementation in captive black rhinoceros diets. The commercial GSE product ActiVin[®] was 64% condensed tannins,

bound 2.56 g of protein per g DM, and had 1.27 g catechin equivalent per g DM iron-binding capacity. Rosé wine GP evaluated in this research was 11.6% proanthocyanidins and had 144 mg catechin equivalent per g of iron-binding capacity. *In vivo* research trials with GSE and GP are necessary to evaluate overall acceptance and effect on dietary iron availability.

INTRODUCTION

Diets of many wild animals contain substantial concentrations of polyphenols, which significantly influence nutrient availability, gastrointestinal and systemic health, and gut microbial populations. Plants produce polyphenols as secondary metabolites involved in diverse processes such as growth, lignification, pigmentation, pollination and resistance against pathogens, predators and environmental stresses (Haslam, 1998; Fraga et al., 2010). These compounds contribute to the color, structure, chemical properties, nutritive value and taste of plant products (Mehansho et al., 1987). Tannins are a classification of polyphenols and are generally described as water-soluble compounds with molecular weights greater than 500 Daltons with the ability to precipitate protein (Hagerman and Butler, 1981).

Polyphenols are a very diverse group, with greater than 8,000 individual compounds identified in various foods typically consumed by humans (Zern and Fernandez, 2005; Perron and Brumaghim, 2009). In the scope of plants foraged by wild herbivores, this diversity increases considerably. Species acceptance and tolerance of tannin concentrations in natural forages is widely varied and inconsistent (Clausen et al.,

1990; Robbins et al., 1991; Helary et al., 2012), and is dependent on multiple biological factors (i.e. salivary tannin binding proteins (Shimada, 2006)), as well as chemical properties of the polyphenolic compound. Historically tannins have been considered “anti-nutritional” compounds which can reduce digestibility of dietary protein, inhibit mineral absorption, and disrupt enzymatic activity (Robbins et al., 1987; Butler and Rogler, 1992). Recent research of tannins in animal nutrition has focused on their many beneficial properties and has begun to elucidate their influence on nutritional physiology and overall animal health (Fraga et al., 2010).

It is important to remember the term “tannin” or even more specific terms cannot be universally applied and compared due to the chemical complexity of these large polyphenolic classes. Tannins are highly polymerized polyphenolic compounds that are generally divided into two classes, hydrolyzable and condensed tannins. Hydrolyzable tannins (**HT**) are esters of a non-phenolic sugar, usually D-glucose, and gallic acid or its derivative (Hagerman, 1992). These compounds hydrolyze easily in acidic or basic conditions to yield the parent sugar and phenolic acids, both of which are readily absorbed.

Condensed tannins are chemically defined as polymerized flavan-3-ols, a subclass of flavonoids (Fraga, 2007), with a basic structure based on a common C6-C3-C6 carbon skeleton. Flavan-3-ols are distinct from other flavonoid classes commonly found in plants because they are present as monomer units (ex. (+)-catechin), gallate derivatives of the monomers (ex. (-)-epigallocatechin gallate, the most abundant tea polyphenol), or as oligomers (i.e. proanthocyanidins or condensed tannins) (Crozier et al., 2006).

Condensed tannins do not undergo hydrolysis in acid or base, but in hot alcohol the flavonoid polymer is oxidatively cleaved to yield colored anthocyanidins, thus these compounds are often called “proanthocyanidins” (Hagerman et al., 1992).

Condensed tannins are characterized based on hydroxylation patterns, stereochemistry, functional groups, interflavan linkages, and degree of polymerization (Ayres et al., 1997; Naumann et al., 2013a). Each of these chemical characteristics relates some specific functionality to the compounds. A flavan-3-ol unit with a tri-hydroxylated B ring is termed a prodelphinidin and a di-hydroxylated B ring is called a procyanidin (ex. the monomer units of the CT in Figure 1-4.) (Foo and Porter, 1980). The procyanidin:prodelphinidin ratio of plant CT, analyzed by HPLC, 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). The type of interflavan linkage may be related to solubility in water (Bate-Smith, 1975). Degree of polymerization into dimers, trimers, tetramers, and higher oligomers can affect its protein precipitation ability (Harbertson et al., 2014; Lorenz et al., 2014). Galloylation at the C3 position increases the potential for hydrogen bonding (Harbertson et al., 2014) and may be related to iron chelation capacity (Andjelkovic et al., 2006). The ability of tannins to bind protein and chelate metal ions are the basic chemical mechanisms underlying many important biological activities.

This diversity of condensed tannin chemical composition complicates analysis and quantification techniques. Several methods have been developed and each has its limitations. However, understanding the underlying principles and limitations as they

relate to tannin chemistry and function may help researchers and nutritionists determine appropriate methods for evaluating the feed value of tannin-containing forages and supplements. Several reviews have discussed the available analytical methods for condensed tannins and their limitations (Makkar et al., 1999; Scho et al., 2001; Pérez-Jiménez et al., 2009; Dai and Mumper, 2010).

Quantitative assays include the Folin-Ciocalteu method for total phenolics (Singleton et al., 1999), the acid butanol (Hagerman, 2002) assay for proanthocyanidins and vanillin assay (Butler et al., 1982) for catechins. These are based on assumptions that structural and chemical characteristics of tannin are met. The protein precipitable phenolics assay is a functional quantification assay for condensed tannins based on the ability of tannins to bind protein (Hagerman and Butler, 1978). Methods to evaluate mineral chelation (Brune et al., 1991; Mladěnka et al., 2011) are also available. High-performance liquid chromatography in conjunction with a UV-visible spectrophotometer, mass spectrometer, or NMR (Stalikas, 2007; Oh et al., 2008) is used to elucidate polyphenolic structural composition. Furthermore, using appropriate standards in each of these assays is critical for accurate quantification. When comparing tannin analyses between studies, the standard compounds used must be considered. Purified CT extracted from the plant or sample of interest (Wolfe et al., 2008) are most accurate and ideal.

These semi-quantitative colorimetric assays (most commonly the acid butanol assay) have been used to measure CT levels in wild herbivore diets. Results suggest that CT concentration of wild black rhinoceros diets is considerably greater than the concentrations of CT in captive black rhino diets, which are provided through

intermittent and opportunistic browse supplementation (Helary et al., 2012). Due to the iron binding ability of polyphenolic compounds, many researchers suggest that browsing rhinoceros and other species susceptible to iron overload disorder (**IOD**) should benefit from the addition of tannins to their diet (Paglia et al., 2001; Gaffney et al., 2004; Clauss et al., 2012; Helary et al., 2012). Lavin (2012) reviewed the use of plant phenolics to mitigate IOD in wild animals and recommended using an iron-binding assay to compare the ability of feedstuffs and/or supplements and browse to minimize available dietary iron in the intestinal lumen. Additionally, recommendations have been made to identify and test palatable, low-cost, available tannin sources for their potential application in preventing excessive iron accumulation in IOD prone species.

OBJECTIVE

The objective of this study was to evaluate grape seed extract and grape pomace tannin functional and chemical characteristics for application in captive black rhinoceros diets to prevent excessive iron absorption.

HYPOTHESIS

Grape seed extract is hypothesized to be a more effective condensed tannin source for supplementation in captive black rhinoceros diets due to greater concentrations of condensed tannins and greater iron binding capacity per unit of dry matter compared to grape pomace.

MATERIALS AND METHODS

Samples and preparation

Commercially available grape seed extract (ActiVin[®]) was donated from San Joaquin Valley Concentrate (Fresno, California). Red grape seeds (*Vitis vinifera*) are harvested and processed using a water only extraction to achieve a crystalized concentrated (>90%) proanthocyanidin product. This specific GSE product was tested because it has passed multiple safety and toxicity studies (Ye et al., 1999; Bagchi et al., 2000; Wren et al., 2013) and is more likely to be a consistent, reproducible product than a less processed grape by-product. Grape pomace was donated from the Oregon Zoo (Portland, Oregon) and was produced in an Eastern Oregon vineyard from red grapes for the production of rosé wine. Vinification of rosé wines involves crushing and pressing the grapes but the skins are not fermented, thus, the majority of proanthocyanidins remain in the pomace. Grape pomace was received by the Oregon Zoo in October 2013, and immediately frozen. Representative samples were sent on dry ice to the University of Missouri and were immediately processed. A subset of whole pomace was hand separated into the skin, seed, and stem fractions.

Grape seed extract was supplied at 95% DM with a particle size < 2 mm so no further preparation was necessary. Whole grape pomace and the skin, seed, and stem fractions were lyophilized (Genesis 25XL, Virtis, Gardiner, NY) and ground with a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA) to achieve particle sizes < 2 mm. Samples were stored at 4°C.

Tannin extraction and purification

Condensed tannins were extracted from 15 g of each sample with 70:30 (vol/vol) acetone:water, followed by a diethyl ether extraction. Tannins were purified from each sample with Sephadex LH-20 (Sigma Aldrich, Saint Louis, MO) using methods adapted from (Strumeyer and Malin, 1975; Naumann, et al., 2013b). The complete tannin purification procedure followed is located in the Appendix (pg. 106). Briefly, each extract was mixed with Sephadex-LH 20 that was equilibrated in methanol and the slurry was washed with methanol until the absorbance of the filtrate reached 280 nm. The tannins were released from the Sephadex by washing with 70:30 (vol/vol) acetone:water. Following vacuum evaporation, purified tannins were lyophilized (Genesis 25XL, Virtis, Gardiner, NY). Dried, purified CT from each sample was used as internally derived standards for GSE and GP in all analyses.

Analysis methods

Acid butanol assay for proanthocyanidins

Method principle

In the acid butanol or butanol-HCl assay, the interflavan bonds between subunits of the flavan-3-ol CT polymers are oxidatively cleaved in hot, acidic alcohol to produce colored anthocyanidins, which are read spectrophotometrically at 550nm (Bate-Smith, 1975). Although this assay is fairly specific for proanthocyanidins, the type of CT (some interflavan bonds are more stable and resistant to cleavage) and assay conditions influence color yields. Water or organic solvents added with the HCl or sample extracts

greatly affects color yield. Iron has been reported to increase color yields, but may not necessarily improve reproducibility of the assay and is not universally used. Heating times also vary between reported methods (10 min to 2 h) and is likely related to the chemical characteristics of the CT being analyzed (Makkar et al., 1999; Grabber et al., 2013).

Reagents

Acetone used for sample tannin extractions is prepared as 70:30 acetone:water. Acid Butanol reagent is prepared by mixing 950 ml of n-butanol with 50 ml concentrated HCl. The ferric ammonium sulfate reagent is prepared by dissolving 0.5 g $\text{FeNH}_4(\text{SO}_4)_2 \times 12 \text{H}_2\text{O}$ in 25 ml of 2 N HCl. Reagent grade methanol is used as supplied.

Procedure

The acid butanol method for determination of extractable and unextractable proanthocyanidins (Hagerman, 2011) was adapted for use with GSE and GP samples and purified CT standards. The entire procedure followed is available in the Appendix (pg. 110). Briefly, 50 mg of dried and ground sample were extracted in triplicate with 70:30 (vol/vol) acetone:water for 30 min. Supernatant was removed and the extraction was repeated two more times. Standard curves were prepared in the acetone mixture using purified CT from GSE and GP, respectively. The resulting three-sample supernatants and standards were mixed in duplicate with 150 μL of methanol, 800 μL of acid butanol, and 33 μL of ferric ammonium sulfate and placed in a boiling water bath (Thermo Scientific,

Precision 280 series, Waltham, MA) for 45 min. Samples were centrifuged (Avanti J-E, Beckman Coulter, Brea, CA) at 1,000 rpm for 5 min. Absorbance of the supernatant was read at 550 nm on a spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA) against a blank of acetone, methanol, acid butanol, and ferric ammonium sulfate.

Protein precipitable phenolics

Method principle

The PPP method quantifies CT by exploiting the ability to bind and precipitate a standard protein. The method of Hagerman and Butler (1978) involves formation of a protein-tannin complex between the tannin-containing solution and bovine serum albumin (**BSA**). The complex is then dissolved using SDS detergent and ferric chloride is added. The reaction of CT with Fe^{3+} forms a purple color and the intensity of color formation is measured spectrophotometrically. Additionally, the amount of bound protein can be quantified to give further insight into the protein binding capacity of tannin containing forages or samples.

Reagents

An acetate buffer (buffer A) of 0.2 M acetic acid and 0.17 M NaCl is adjusted to pH 4.9. A BSA protein standard solution is prepared by mixing 5 mg BSA/ml of buffer A. The detergent solution used to dissociate the protein-tannin complex is a 1% sodium dodecyl sulfate (**SDS**) and 5% triethanolamine (**TEA**, $\text{C}_6\text{H}_{15}\text{NO}_3$) solution in deionized water. The iron reagent is a filtered solution of 0.01 M FeCl_3 in 0.01 M HCl.

Procedures

Protein precipitable phenolics

The scaled-down method described by Hagerman and Butler (1978) was followed to determine PPP. The complete procedure is described in the Appendix (pg. 113). To summarize, samples were extracted in triplicate one time as described for the acid-butanol procedures. Supernatant from each sample was assayed in duplicate by addition of buffer A, BSA solution, and 50:50 (vol/vol) methanol:water to create a protein-tannin complex, which is pelleted through centrifugation. The pellet was dissolved in the SDS/TEA detergent solution, thus dissociating the protein-tannin complex. Released tannins were then available to bind with Fe_{3+} after addition of the iron reagent. The reaction results in purple color and the intensity of color formation was measured spectrophotometrically at 510 nm and compared to a standard curve (created using purified GSE and GP condensed tannin standards) for condensed tannin quantification.

Quantification of bound protein

To quantify protein bound by the CT, methods described by Naumann et al. (2014) were followed using purified GSE condensed tannins. Precipitation of BSA was performed as described above. Instead of dissolving the protein-polyphenol complex, the BSA-CT pellet was vortexed in 500 μ L of Buffer A and the solution was transferred into a pre-weighed foil cup dried in a forced air oven (Isotemp Oven 200 series, model 255 G, Fisher Scientific, Pittsburgh, PA). The dried protein-CT residue was analyzed for N

(Vario Macro Cube, Elementar Americas, Mt. Laurel, NJ) to quantify precipitated protein. Percent nitrogen was multiplied by 6.25 to convert to crude protein.

Iron-binding capacity

Method summary

Iron-binding capacity was measured using the method developed by Brune et al. (1991), with some modifications. Phenolic compounds, extracted by dimethylformamide (DMF) in an acetate buffer, react with iron and form a color that is read spectrophotometrically at two wavelengths, 578 and 680 nm. The absorbencies, measured against a blank, correspond to formation of iron-galloyl complexes (blue color, 578 nm) and iron-catechol complexes (green color, 680 nm).

Reagents

A 1% acetate buffer was prepared by combining 0.1 N acetic acid and 0.1 N sodium acetate solutions and was adjusted to pH 4.4. The DMF-acetate solution was prepared by mixing equal volumes of DMF and 1% acetate buffer. A gum arabic solution was prepared by solubilizing 1 g in 100 ml deionized water and a 5% ferric ammonium sulfate (FAS) solution was prepared in 1% HCl. The FAS reagent was prepared by mixing 89 parts 1% acetate buffer, 10 parts gum arabic solution, and 1 part FAS solution. A food blank reagent was prepared the same as the FAS reagent but the FAS solution was replaced with 1% HCl.

Procedure and calculations

The complete procedure, reagent recipes, and calculations are described in the Appendix (pg. 117). To summarize, 500 mg of each sample were extracted with 50 ml of the DMF-acetate solution for 16 h at room temperature. Samples were centrifuged at 3500 x g for 15 min and the supernatant was used for analysis. Standard curves were prepared by serial dilution of tannic acid and (+)-catechin standards (Sigma Aldrich, Saint Louis, MO) corresponding to absorbance at 578 nm and 680 nm, respectively. One set of sample supernatant was mixed in duplicate with FAS reagent and a second set was mixed in duplicate with the food blank reagent.

Each set of samples was measured spectrophotometrically against a blank at 578 and 680 nm. The sample absorbance was determined by subtracting the sample “food blank” absorbance from the sample FAS absorbance. The concentrations of catechol and galloyl groups were calculated by using the linear regression equation from the standard curves of tannic acid and (+)-catechin hydrate at the two respective wavelengths.

HPLC analysis of anthocyanidin monomers

Method summary

High-performance liquid chromatography (**HPLC**) is the method of choice for anthocyanidin determination because this technique allows efficient separation, identification, and quantification of anthocyanidins in CT-containing samples. Reactions described in the acid-butanol method summary (section 1.1) are used to depolymerize the CT into anthocyanidin monomers. The methods utilized in this research do not allow

degree of polymerization estimation, but do determine anthocyanidin composition and relative concentrations in CT samples.

Procedure

Solutions of purified condensed tannins from GSE and GP were prepared at 25 mg/ml of 95% butanol 5% HCl. The GP solution and one GSE solution were allowed to react for 45 min in a boiling water bath. A second GSE solution was allowed to react for 24 hours to detect if any interflavan bonds were resistant to breaking when reacted for only 45 minutes. Anthocyanidin monomer analysis was conducted on the products of the acid butanol reaction using HPLC analysis (Thermo Scientific™ Dionex™, Sunnyvale, CA) controlled by Chromeleon™ Software (Thermo Scientific™ Dionex™, Sunnyvale, CA). A 3.0 mm x 150 mm, 2.6 μm Thermo Scientific™ Accucore™ C₁₈ column was used. Prior to injection, the 30°C column was equilibrated for 5 min with 5% mobile phase B (1 ml/L trifluoroacetic acid in acetonitrile (MeCN)). Five μl of each sample were 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 analyzed 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.

RESULTS

Acid butanol assay for proanthocyanidins

As expected, grape seed extract has greater proanthocyanidin concentration than whole grape pomace (429.65 vs. 116.33 mg CT/g DM, respectively). Results from the acid butanol analysis of GSE and GP for proanthocyanidins are presented in Table 2-1.

Table 2-1. Proanthocyanidin concentration (mg condensed tannin (CT)/g dry matter (DM)) in grape seed extract and whole grape pomace as determined by the acid butanol assay.

Sample	mg CT / g DM	SD
Grape Seed Extract	429.65	44.06
Grape Pomace - whole	116.33	15.10

Protein precipitable phenolics

Condensed tannin concentration determined by protein precipitation resulted in greater values for GSE than the acid butanol assay, but not for whole grape pomace. Of the three components of whole grape pomace, the seeds had the greatest CT concentration (123.28 mg/g DM) followed by the stems (41.03 mg/g DM) and grape skins (22.37 mg/g DM). Results from the protein precipitable phenolics analysis of GSE, whole GP, GP stems, GP seeds, and GP skins are presented in Figure 2-1.

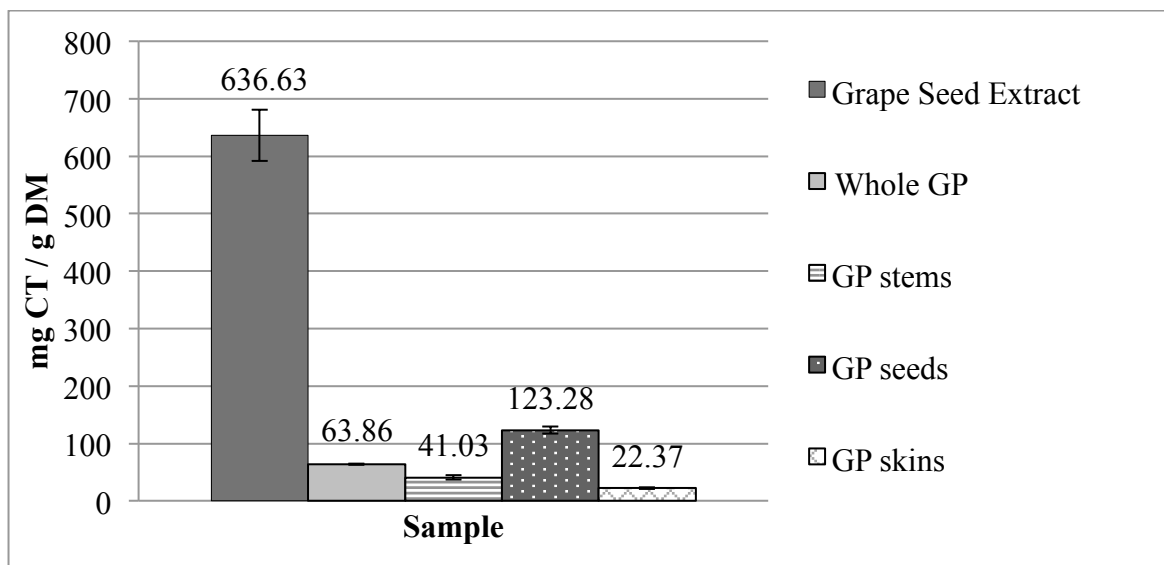


Figure 2-1. Concentration of protein precipitable condensed tannins (CT) per g dry matter (DM) in grape seed extract, whole grape pomace (GP), and grape pomace stems, seeds and skins.

Quantification of bound protein

The amount of BSA protein bound per g of purified GSE CT averaged $4,592.33 \pm 521.90$ mg. This value multiplied by the average GSE CT concentration (determined by PPP method) estimates that about 2.56 g of protein are precipitated per g of dried GSE.

Iron-binding capacity

The iron-binding capacity of GSE and GP was tested to estimate potential effectiveness of the CT sources as iron chelators in captive black rhinoceros diets. Tannic acid and catechin were used as the standards as proposed by Brune et al. (1991). Both samples demonstrated large differences between tannic acid equivalents (TAE) and (+)-catechin equivalents (CE) (Figure 2-2). Results suggest GSE had 8 – 8.8 times greater iron-binding capacity than GP, which is expected considering greater polyphenolic

concentration in GSE. When sample iron-binding capacity (mg CE/g DM) was normalized for proanthocyanidin concentration (mg proanthocyanidin/g DM), grape seed extract still demonstrated greater iron-binding capacity (Table 2-2).

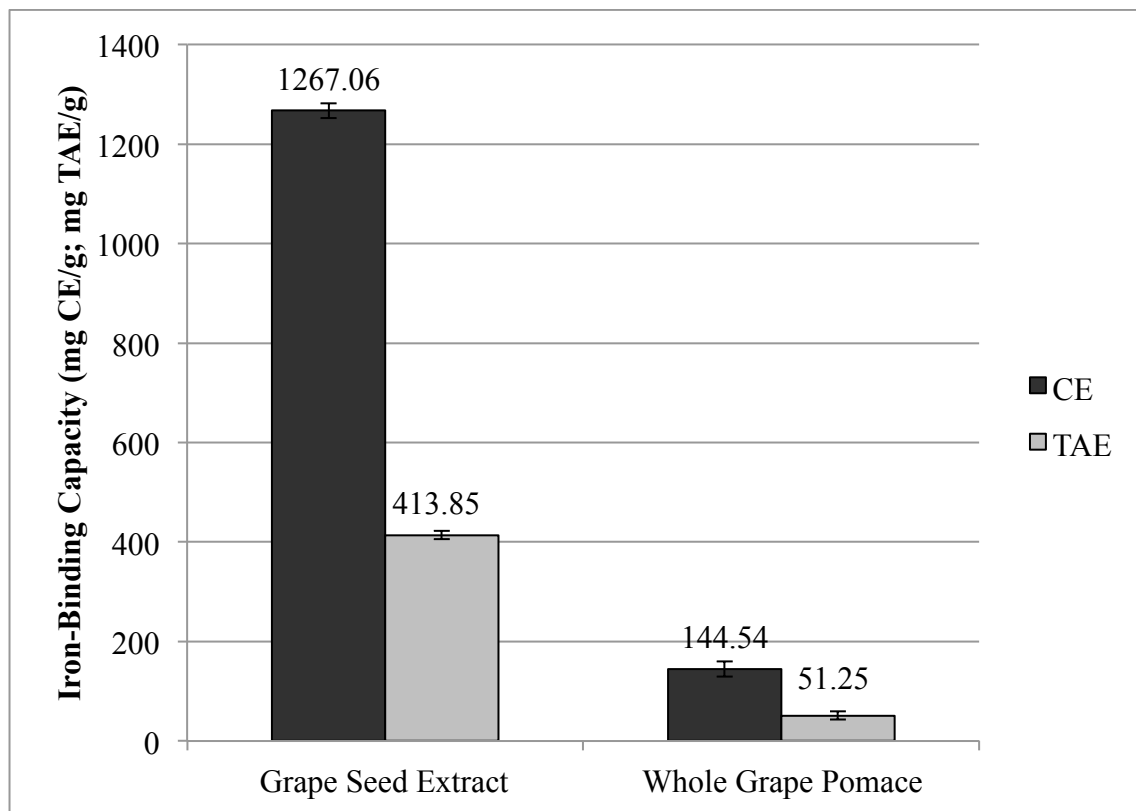


Figure 2-2. Tannin iron-binding capacity of grape seed extract and whole grape pomace. (+)-Catechin equivalents (CE) and tannic acid equivalents (TAE) per g dry matter are presented for both samples.

Table 2-2. Iron-binding capacity (mg (+)-catechin equivalents (CE)) of grape seed extract and whole grape pomace per mg proanthocyanidin (determined using the acid butanol assay) or per condensed tannin (determined by the protein precipitable phenolics method).

Sample	mg CE / mg proanthocyanidin	mg CE / mg CT
Grape Seed Extract	2.95	1.99
Whole Grape Pomace	1.24	0.02

HPLC analysis

Purified GSE and GP condensed tannins were analyzed for anthocyanidin composition using HPLC. Both samples were predominately composed of cyanidin monomers, but GSE had greater cyanidin to delphinidin ratio and a greater proportion of gallated monomers (0.60 vs. 0.47) compared to GP (Table 2-3). A reaction time of 24 h in boiling butanol-HCl was predicted to cleave interflavan bonds that might have been resistant when only reacted for 45 min. However, anthocyanidin composition was similar between samples reacted at 45 min and 24 h (Table 2-4). Gallic acid concentrations increased in samples reacted for 24 h compared to 45 min (Table 2-4).

Table 2-3. Cyanidin to delphinidin ratio and proportion of gallated monomers of grape seed extract and grape pomace.

Sample	cyanidin:delphinidin	Proportion gallated
Grape Seed Extract	14.0:1.0	0.60
Grape Pomace	8.5:1.0	0.47

Table 2-4. Anthocyanidins measured (mg/ml) in grape seed extract after 45 min and 24 h of 95 °C acid butanol reaction.

Grape Seed Extract	Anthocyanidin Monomer (mg/ml)			Gallic Acid
	Delphinidin	Cyanidin	Pelargonidin	
45 min reaction	0.0001	0.0014	-	0.0009
24 h reaction	0.0001	0.0013	-	0.0015

DISCUSSION

Grape seed extract is a more concentrated source of condensed tannins than grape pomace. Although both are derived from red grapes, results indicate that polyphenolic composition may vary between GSE and GP. Estimation of GSE CT concentration was greater using the PPP method (637 mg/g) compared to the acid butanol assay (430 mg/g), whereas the opposite trend was observed for GP (PPP = 64 mg/g, acid butanol = 116 mg/g). The PPP method is based on the ability of extracted tannins to precipitate protein and this ability has been positively correlated with CT polymer length (Hagerman, 2012; Harbertson et al., 2014; Lorenz et al., 2014). Comparatively, the acid butanol assay relies on detection of monomer anthocyanidin units after the CT has presumably been depolymerized. These results may indicate that GSE CT have a greater average degree of polymerization, resulting in comparatively stronger protein-binding ability per unit of CT than GP. Greater acid butanol values (rather than PPP) for GP, supports this hypothesis since smaller polyphenolic units are more easily cleaved and result in faster and more intense color development in the assay. Magenta color development was observed within seconds of adding the acid butanol reagent, whereas color development for GSE and other samples required much longer reaction times in the boiling water bath. Thus, it is possible that GP proanthocyanidins are smaller polymer units compared to GSE, resulting in greater estimation of CT in the acid butanol assay for GP and in the PPP assay for GSE CT. These results suggest that if GSE and GP were supplemented at equivalent proanthocyanidin concentrations in a diet, GSE might result in a greater reduction of protein digestibility than GP.

A key limitation of the acid butanol reaction is the varying stability or reactivity of interflavan bonds (Scho et al., 2001). Interflavan bonds commonly associated with quebracho tannins have low reactivity in the assay, yielding less intense color formation at high concentrations. When quebracho CT are used as a standard in the assay, concentrations of CT composed of reactive interflavan bonds are over estimated. This effect has been observed in two ecotypes of *Acacia angustissima* var. *hirta* (prairie acacia), which demonstrate moderate CT protein-binding but are almost completely unreactive in butanol-HCl (Naumann et al., 2014). To test for the presence of unreactive interflavan bonds in GSE, samples were reacted with butanol-HCl for either 45 min or 24 h. HPLC analysis showed no difference in cyanidin or delphinidin concentration (Table 2-4) indicating that all potentially reactive interflavan bonds have been cleaved by 45 min. However, there was one unidentified peak in the chromatogram. This peak is likely a different anthocyanidin for which we did not have a standard, but could potentially represent CT polymers unreactive in acid-butanol.

In both samples, there were large differences between iron-binding capacity as TAE and CE (Figure 2-2). Greater iron-binding values as catechin equivalents indicate that both samples primarily contained condensed tannins, although some iron binding by galloyl groups was detected (TAE). This was confirmed by HPLC analysis. Both GSE and GP had much greater concentrations of cyanidin than delphinidin (catechol vs. galloyl B-ring hydroxylation patterns) and had moderate proportions of gallated monomers (Table 2-3). Presumably due to greater proanthocyanidin concentration, GSE demonstrated 8.7 times greater iron-binding capacity than GP (Figure 2-2). When CE

were normalized on proanthocyanidin concentration (determined by the acid butanol method), GSE still had 2.38 times greater iron-binding capacity than GP (Table 2-2). Increasing free hydroxyl groups generally improves CT-metal ion chelation (Andjelkovic et al., 2006; Mladěnka et al., 2011). Although more delphinidin moieties were identified in GP samples, GSE had a higher proportion of gallate monomers (Table 2-3), which may contribute to improved iron-chelation ability.

Spradling (2008) compared iron-binding capacities of five Missouri grape pomace varieties using the same methodologies used in this experiment. Pomace from the Vincent variety, used for rosé wine production, had the greatest iron-binding capacity and measured about 120 mg CE/g. The grape pomace variety in this experiment, also from rosé wine production, had 144 mg CE/g indicating it may be a more efficient iron-chelation supplement than the Vincent variety.

CONCLUSION

In support of our hypothesis, grape seed extract was found to be a more effective source of CT for supplementation in captive black rhinoceros diets. The commercial GSE product ActiVin[®] was 64% CT, bound 2.56 g of protein per g DM, and had 1.27 g catechin equivalent per g DM iron-binding capacity. Rosé wine GP (2013) evaluated in this research was 11.6% proanthocyanidins and had 144 mg catechin equivalent per g DM of iron-binding capacity. However, economic costs must be taken into consideration and if GP from rosé wine production is available it will likely be a more feasible choice

for supplementation in IOD-prone rhinoceros diets. *In vivo* research trials with GP and GSE are necessary to evaluate overall acceptance and effect on dietary iron availability.

CHAPTER 3

COMPARING BLACK RHINOCEROS (*DICEROS BICORNIS*) AND DOMESTIC HORSE (*EQUUS CABALLUS*) HINDGUT MICROFLORA FERMENTATION RESPONSES TO GRAPE SEED EXTRACT SUPPLEMENTATION

ABSTRACT

As large hindgut fermenting herbivores, the gastrointestinal anatomy of rhinoceros most resembles that of equids and the domestic horse (*Equus caballus*) has frequently been used as a nutritional model for all rhinoceros species. Iron chelators, such as tannins, are currently under investigation as supplements to ameliorate or prevent iron overload disorder in black rhinoceros (*Diceros bicornis*). Variable effects of polyphenolic compounds on microbial fermentation have been reported, making it imperative to determine potential tannin supplement effects on black rhinoceros fermentation. Equine *in vivo* studies are 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 objectives of this experiment were to compare fermentation characteristics and nutrient digestibility between the black rhinoceros and domestic horse and to examine grape seed extract (GSE; a concentrated source of tannins) effects on fermentation parameters using a continuous single-flow *in vitro* culture system. Two replicated

continuous culture experiments were conducted using domestic horse and black rhinoceros feces as inoculum sources comparing four diets with increasing GSE inclusion (0-4% of dry matter). Increasing GSE inclusion stimulated microbial growth and fermentation. Domestic horse and black rhinoceros hindgut microflora nutrient digestibility and fermentation responses to GSE did not differ. Contrary to our hypothesis, 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 nutrient digestibility or microbial viability and fermentation.

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 has frequently been used as a nutritional model for all rhinoceros species (Ofstedal et al., 1996; Dierenfeld, 1999; Miller, 2003; Clauss et al., 2006a). The adequacy of this model has been confirmed for grazing rhinoceros species (African white (*Ceratotherium simum*) and Indian (*Rhinoceros unicornis*)) (Frape et al., 1982; Clauss et al., 2005a) and was found to be adequate for captive tapir nutrition as well (Clauss et al., 2009). However, inadequacies of the horse model have been reported for elephants (Ofstedal et al., 1996). The high prevalence and

severe health consequences of iron overload suggests a need to reevaluate the domestic horse model for browsing rhinoceros nutrition.

Fermentation of structural carbohydrates and undigested dietary components occurs in the cecum and colon of hindgut fermenting herbivores, with the products of fermentation (volatile fatty acids (VFA)) significantly contributing to overall energy metabolism of the animal (Glinsky et al., 1976; Harris, 1997; Santos et al., 2011). Cecal fermentation has never been compared between browsing rhinoceros and horses but may explain differences observed in digestibility of similar diets between the two species. Fundamental differences in digestive physiology between grazing and browsing species suggests fermentation may differ (Clauss et al., 2005b; Clauss et al., 2006a; Hummel et al., 2006). Equine *in vivo* studies are often used to assess diets or supplements for rhinoceros, therefore, evaluating the use of a domestic horse model for black rhinoceros fermentation is crucial.

Iron chelators, both synthetic and natural (i.e. polyphenolic compounds), are currently under investigation as supplements to ameliorate or prevent iron overload disorder in black rhinoceros. Variable effects of polyphenolic compounds on microbial fermentation have been reported, and results appear to depend on type and concentration (Tebib et al., 1996; Martin-Carron and Goni, 1998). Gut microflora may depolymerize condensed tannins (CT) producing small, bioavailable proanthocyanidins, or may catalyze ring fission reactions (Deprez et al., 2000). Historically, tannins have been regarded as inhibitory to the growth of microbes (Min et al., 2006; Patra and Saxena, 2011) due to mechanisms including membrane disruption, enzyme inhibition, and

substrate limitation (Smith et al., 2005; Kemperman et al., 2010). Although some bacterial populations may be inhibited, others can thrive (Kemperman et al., 2010). For example, increased cecal microbial diversity was observed in chickens fed grape pomace and GSE (Viveros et al., 2011). Supplementation with condensed tannins, but not monomers, has been associated with increased VFA concentration in rat cecum at 7% of diet DM (Bravo et al., 1994; Tebib et al., 1996). However, there was no increase in fecal VFAs when fed at 1.5% of dry matter (**DM**) to black rhinoceros (Clauss et al., 2007a). This variation makes it imperative to determine potential CT supplement effects on black rhinoceros microbial populations, fermentation characteristics, and overall nutrient digestibility.

OBJECTIVES

The objectives of this experiment were to compare fermentation characteristics and nutrient digestibility between the black rhinoceros and domestic horse and to examine grape seed extract effects on fermentation parameters using a continuous single-flow *in vitro* culture system.

HYPOTHESIS

We hypothesized fermentation characteristics and response patterns to GSE inclusion would differ between the horse and black rhinoceros due to differences in typical diet composition and digestive physiology between grazing and browsing species (Hummel et al., 2006).

MATERIALS AND METHODS

Experiment diet and treatments

The basal diet was based on average composition of black rhinoceros diets at the Saint Louis Zoo (Saint Louis, MO). Basal diet ingredients were ground to pass through a 2-mm screen (Wiley mill, Arthur H. Thomas Company, Philadelphia, PA) and mixed (Hobart, Troy, Ohio) prior to GSE inclusion. Dietary treatments consisted of four GSE (ActiVin[®], San Joaquin Valley Concentrate, Fresno, CA) concentrations added to the basal diet (Table 3-1). Grape seed extract inclusion replaced total diet.

Table 3-1. Basal diet for continuous culture experiments and grape seed extract (GSE) treatment levels.

Ingredient	Percent of basal diet ¹
Orchard grass/Alfalfa Hay	83.05
High Fiber Pellets ²	15.96
Kale	0.48
Carrots	0.41
Apple	0.10
Treatment	GSE ² inclusion level (Percent of diet DM)
Control	0.00
Low	1.33
Mid	2.66
High	4.00

¹ Basal dietary ingredients were dried at 95°C, ground to 2 mm, and mixed prior to grape seed extract (GSE) inclusion. GSE replaced total diet. Ingredient composition presented on a dry matter (DM) basis.

² High fiber pellets (ADF-25, Mazuri[®] Exotic Animal Nutrition, PMI Nutrition, Saint Louis, MO)

³ GSE (ActiVin[®], San Joaquin Valley Concentrate, Fresno, CA)

Animals and fecal collection

Two adults (1 male, 1 female) and one juvenile male East African black rhinoceros (*Diceros bicornis michaeli*), housed at the Saint Louis Zoo, were fed their normal diet (study basal diet) but were removed from browse and enrichment food items containing polyphenolics for 14 days prior to fecal collection. The Saint Louis Zoo Animal Biomaterials Sample Committee approved this protocol and collection of black rhinoceros feces for this experiment. Three adult female quarter horses (*Equus caballus*), housed at the University of Missouri Equine Teaching and Research Farm (Columbia, MO) were transitioned to the basal diet over a 5 d period and were adapted to the basal diet for 14 d prior to fecal collection. The University of Missouri Animal Care and Use Committee approved the use of horses for this experiment. Hay fed to the horses during the adaptation period and used for both continuous culture experiments was donated by the Saint Louis Zoo, and came from the same source and lot as the hay fed to the rhinoceros prior to fecal collection.

Following the 14-d adaptation periods, total feces were collected within 30 min of excretion and the outer layer was removed to prevent contamination. Feces were mixed with a glycerol-McDougall's Buffer solution at 20% (vol/wt), flushed with CO₂ and immediately frozen on dry ice for transportation. Samples were stored at -20°C until use. The detailed fecal collection protocol is available in the Appendix (pg. 119).

Continuous culture procedures and analyses

Prior to inoculating the continuous culture, fecal samples were thawed and pre-incubated based on methods described by Luchini et al. (1996). Equal proportions of feces from each animal, within species, were combined, mixed with equal parts (wt/vol) of a pre-incubation media, and incubated at 39°C for 6 h. All necessary precautions to prevent microbial contamination between species was taken.

Two replicated continuous culture experiments were conducted using domestic horse and black rhinoceros feces as inoculum to compare four diets with increasing GSE inclusion. Complete continuous culture experimental procedures can be found beginning on page 120 of the Appendix. Briefly, twenty-four single-flow continuous culture vessels (Nalgene, Rochester NY) were inoculated (12 per species) and maintained as described by (Meng et al., 1999). Fermenters were continually flushed with CO₂, stirred with magnetic stir plates (Thermix Stirrer model 120S, Fisher Scientific, Pittsburgh, PA), and incubated in a 39 °C water bath using thermostatically controlled heaters (model 730, Fisher Scientific, Pittsburgh, PA). A buffer solution was continuously added to the fermenter containers using calibrated peristaltic pumps (Masterflex model 7520-10, Cole Palmer Instrument Co., Chicago, IL) that maintained a constant dilution rate of 3.8% h⁻¹. Effluent was collected in vessels immersed in an ice bath. Diets (n=6 fermenters per treatment per run) were fed twice daily at 12 h intervals.

The following conditions were used to accurately represent hindgut fermentation using the *in vitro* continuous culture model. To mimic the form of feed typically presented to cecal and colonic bacterial populations, a simulated foregut digestion

procedure was used according to methods described by Sunvold et al. (1995), Boisen and Fernhdez (1997), Murray et al. (2007), and Sweeney (2012). The detailed procedure is described in the Appendix (pg. 128). Briefly, diet samples (n=10/treatment) were subjected to an artificial saliva incubation (including α -amylase), followed by acidic pepsin digestion. Digesta pH was then increased to 6.8 and samples were incubated with pancreatic enzymes, washed with deionized water to remove solubilized nutrients, and dried in a 55°C forced air oven (model 7921, Blickman Health Industries, Clifton, NJ). Unfortunately, this digestion procedure was far too time and labor intensive to be feasibly implemented and compatible with the continuous culture apparatus. However, nutrient foregut *in vitro* digestibility was measured and compared among GSE treatments (n=10 per treatment). Diets were fed in their original dried and undigested form throughout both continuous culture experiments. Thirty-five g of diet were fed per day, equally split between two feedings at 12 h intervals. Dietary nutrient composition is displayed in Table 3-2.

Table 3-2. Continuous culture diet nutrient composition¹

Treatment ²	% DM	% OM	% CP	% NDF	% ADF	Iron (ppm)
Control	93.17	90.94	20.72	42.80	28.08	271.82
Low	94.39	91.24	20.82	42.26	27.55	232.78
Mid	94.46	91.24	20.43	41.75	26.87	242.69
High	94.32	91.34	20.18	42.12	27.47	243.59
Average	94.08	91.19	20.54	42.23	27.49	247.72

¹ Nutrient composition presented on a dry matter (DM) basis: organic matter (OM); crude protein (CP); neutral detergent fiber (NDF); acid detergent fiber (ADF); iron

² Dietary treatments: Control = basal diet; Low = basal diet + 1.33% grape seed extract (GSE); Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE

Sampling

Experiments were conducted over 8-d periods with 5 adaptation days and 3 sampling days. Fermenter samples were taken and pH was measured at 0, 2, 4, 6 and 8 hours relative to feeding and stored at -20°C until analysis for VFA and ammonia (NH_3) concentration. Total effluent contents were collected over 24 hours of each sampling day and stored at 4°C until further analysis. All samples were composited over the 3 sampling days. At the end of each experiment, fermenter contents were blended, strained through 4 layers of cheese cloth, and stored at 4°C until analysis.

Laboratory analysis

Blended fermenter contents were centrifuged at 1,000 x g for 5 min at 4°C to remove feed particles. Supernatant was re-centrifuged at 27,000 x 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 bacterial pellets were lyophilized (Genesis model 25XL, Virtis, Gardiner, NY) until 95% dry and ground using a mortar and pestle.

Diet and effluent samples were analyzed for DM, organic matter (**OM**), and crude protein (**CP**) concentration. Due to a lack of consensus on sodium sulfite inclusion for fiber determination in tannin containing samples, diet and effluent samples were analyzed for fiber components (neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**)) both with and without the addition of sodium sulfite (**SS**). Values for NDF and ADF, as well as fiber digestibility, were compared between the two methods. Effluent and bacteria

samples were analyzed for purine content using the procedure of Zinn and Owens (1986) to calculate microbial nitrogen. Microbial nitrogen production and OM digested were used to calculate microbial efficiency (**MOEFF**; g microbial nitrogen outflow/kg OM truly digested).

Diet and effluent samples were also analyzed for condensed tannin concentration following the protein precipitable phenolics (**PPP**) method described by Hagerman and Butler (1978). Furthermore, diet samples were analyzed for proanthocyanidin concentration (Hagerman, 2011) and iron-binding capacity (Brune et al., 1991). Condensed tannins purified from the high GSE diet (Strumeyer and Malin, 1975; Naumann, et al., 2013b) were used as standards in all tannin assays and were characterized by HPLC analysis for anthocyanidins. Tannin methods and procedures used in this experiment followed the protocols described in Chapter 2.

Fermenter samples taken at multiple time points throughout the experiment were compiled by hour, across the three sampling days. These samples were analyzed spectrophotometrically (Evolution 201, Thermo Scientific, Waltham, MA) for NH₃ concentration following the phenol hypochlorite method of (Broderick and Kang, 1980). Sample VFA concentration was measured using gas chromatography (430 Gas Chromatographer, Bruker Corporation, Fremont, CA) following the procedures of Galyean and May (2010: p160–162).

Statistical analyses

This experiment was conducted as a randomized complete block, with a 2 x 4 factorial design, to analyze the effects of inoculum source (**species**; horse vs. rhino) and GSE inclusion (**treatment**; Control, Low, Mid, or High). Experimental replication (continuous culture run) was the blocking variable. All statistical analyses were performed using SAS[®] version 9.4 (SAS Institute Inc., Cary, NC). True nutrient digestibility, microbial organic matter (OM_m) production, and MOEFF analyses were conducted using the GLM procedure, with fermenter as the experimental unit. The Mixed procedure was used to analyze VFA, NH₃ 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 significant ($P \leq 0.05$), mean separation was performed using Fisher's Least Significant Difference. Linear, quadratic and cubic contrasts were tested for the effect of GSE treatment. The Correlation procedure was used to analyze relationships among the three assays utilized for tannin characterization.

RESULTS

Tannin analysis

Acid butanol assay for proanthocyanidins

Extractable and unextractable proanthocyanidin (**PA**, i.e. condensed tannin) concentrations were determined separately for each continuous culture. No PAs were detected in any sample residue following three, 30-min acetone extractions (Unextractable PA, Table 3-3) indicating that GSE tannins and those intrinsic in the basal

diet are readily extractable. Extractable PA concentration increased with increasing GSE inclusion and values for each treatment were similar between both continuous cultures (Table 3-3). The High GSE diet had the greatest PA concentration (60 mg/g DM). Although no GSE was supplemented in the control diet, PAs were detected (3.31 mg/g DM) demonstrating low inherent tannin concentrations in the basal diet.

Table 3-3. 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 ¹	CC run ²	mg extractable ³ CT / g sample	SD ⁴	mg unextractable ³ CT / g sample
Control	1	2.90	0.381	-0.01
	2	3.72	0.654	-0.01
Low	1	21.14	4.120	0.00
	2	27.58	5.518	0.00
Mid	1	41.29	7.725	0.00
	2	49.28	8.190	0.00
High	1	58.27	5.265	0.00
	2	62.36	4.226	0.00

¹ 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

² Two replicated continuous culture (CC) experiments were conducted. Diets were mixed separately for each continuous culture.

³ Extractable CT = CTs measured in the supernatant after samples were extracted 3 times in 70% acetone. Unextractable CT = CTs remaining in the sample after 3 acetone extractions.

⁴ Sample standard deviation (assayed in triplicate)

Protein precipitable phenolics

Results from the PPP assay are presented in Figure 3-1. Similar to acid butanol results, the High diet had the greatest CT concentration (26.3 mg CT/g DM) and the Control diet had the lowest, yet still detectable concentrations (0.7 mg CT/g DM). Condensed tannin concentration determined by protein precipitation resulted in lower values for all diets than the acid butanol assay. However, the magnitude of CT

concentration increase with increasing diet GSE inclusion was similar. Acid butanol and PPP assays were tightly correlated ($P < 0.0001$, $R^2 = 0.97$).

Effluent content was measured for condensed tannin content using the PPP method to assess whether microbial metabolism or some alteration of GSE tannins occurred. No detectable levels of CT were measured in any effluent sample from any treatment for either experiment.

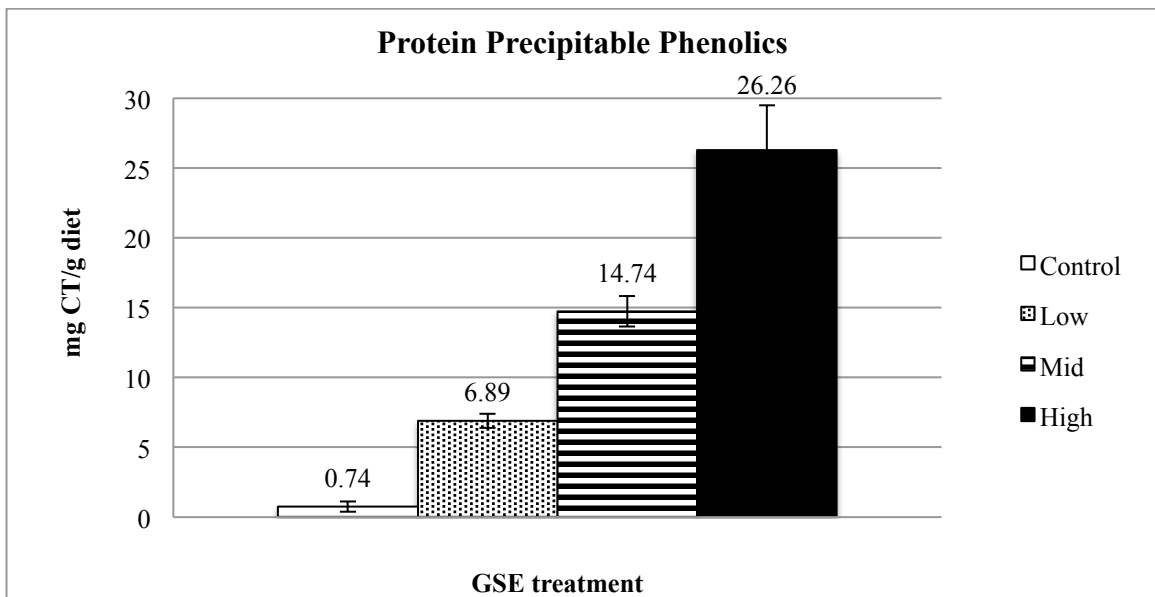


Figure 3-1. Protein precipitable condensed tannins (CT) concentration (mg) per g of continuous culture diet dry matter. Dietary treatments consisted of Control = basal continuous culture diet; Low = basal diet + 1.33% grape seed extract (GSE); Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE.

Quantification of bound protein

The amount of BSA protein bound per gram of purified diet CT averaged $4,094.42 \pm 668.800$ mg. This value multiplied by the average CT concentrations

(determined by PPP method) estimates that about 3.03, 28.23, 60.35, and 107.5 g of protein can be precipitated per kg of Control, Low, Mid and High diet DM, respectively.

Iron-binding capacity

The iron-binding capacity of each diet was tested to estimate potential effectiveness for reducing free iron availability in captive black rhinoceros diets. Results from both continuous culture diets were averaged within treatment and are presented in Figure 3-2. Tannic acid and catechin were used as standards for quantifying iron-binding capacity as proposed by Brune et al. (1991). Samples demonstrated large differences between tannic acid equivalents (TAE) and (+)-catechin equivalents (CE), and the magnitude of this difference increases as grape seed extract inclusion increases. Greater iron-binding values as catechin equivalents indicate that diets primarily contained condensed tannins, although some iron binding by galloyl groups was detected (TAE). This was confirmed by HPLC analysis. Tannin concentrations (CE) determined by the iron-binding method were similar to proanthocyanidin concentrations determined in the acid butanol assay for Mid and High diets. However, iron-binding CE were greater than PA concentration for Control and Low diets (15.57 and 30.98 mg CE/g vs. 3.31 and 24.36 mg CT/g). Regardless, iron-binding capacity results were tightly correlated with the acid butanol assay ($P < 0.0001$, $R^2 = 0.97$) and the PPP method ($P = 0.0002$, $R^2 = 0.96$).

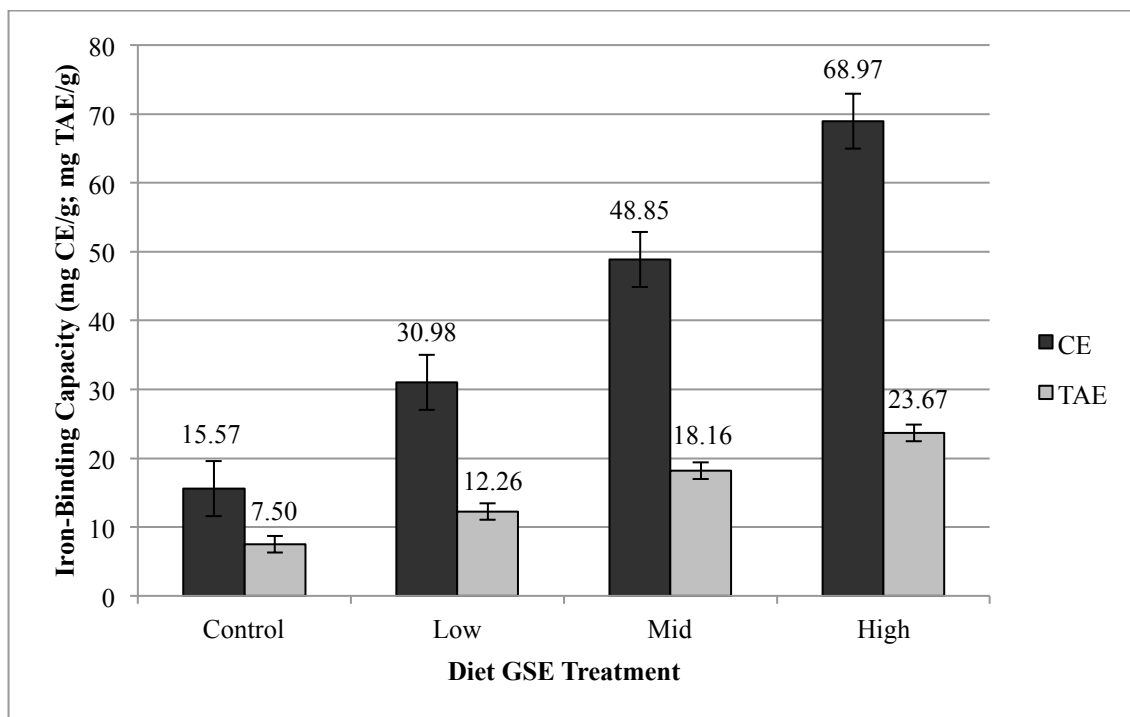


Figure 3-2. Continuous culture diet iron-binding capacity. (+)-Catechin equivalents (CE) and tannic acid equivalents (TAE) per g dry matter are presented for both samples. Dietary treatments consisted of Control = basal continuous culture diet; Low = basal diet + 1.33% grape seed extract (GSE); Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE

HPLC analysis

Purified High diet CT anthocyanidin composition (Table 3-4) was similar to that of purified GSE (results presented in Chapter 2). However, there were slightly greater delphinidin and cyanidin concentrations detected in continuous culture diet CT. Cyanidin:delphinidin ratio and proportion of gallated monomer units were lower in diet CT compared to purified GSE (0.47 vs. 0.60). These results corroborate the acid butanol, PPP, and iron-binding assay results, which suggest a low inherent tannin concentration in the basal continuous culture diet, before the addition of GSE.

Table 3-4. High performance liquid chromatography (HPLC) analysis of anthocyanidin monomers in purified continuous culture diet condensed tannins (CT).

HPLC Anthocyanidin Monomer analysis				
Sample	Monomer Concentration (mg/ml)			
	Delphinidin	Cyanidin	Pelargonidin	Gallic Acid
	0.0002	0.0017	-	0.0009
Purified diet CT	CT monomer composition			
	cyanidin:delphinidin		proportion gallated	
	8.5:1.0		0.47	

Effect of sodium sulfite on fiber analysis of tannin containing samples

Diet and effluent samples were analyzed using both NDF methods (with and without SS) followed by sequential ADF determination. Both diet and effluent sample NDF and ADF values were greater ($P \leq 0.05$) when determined without SS (Table 3-5). As there is no reason to believe SS is solubilizing fiber components (cellulose, hemicellulose, cell wall protein or lignin) then the lower values are likely more accurate.

Diet and effluent NDF and ADF values are used to calculate fiber digestibility in continuous culture fermentation experiments. NDF digestibility was greater ($P = 0.0012$), and ADF digestibility tended to be greater ($P = 0.0945$), when calculated using SS-included NDF and ADF values (Table 3-5). No interactions between SS inclusion and dietary treatments (with differing CT concentrations) were measured in any of the three data sets ($P \geq 0.15$). Our results suggest that for the CT-containing diets used in this study, fiber values determined using SS are more accurate. Therefore, all NDF and ADF results presented in this paper have been calculated using published ANKOMTM detergent fiber analysis procedures with the addition of α -amylase and sodium sulfite.

Table 3-5. Effect of sodium sulfite (SS) on sample fiber concentration (percent of dry matter) and digestibility calculations.

Sample Type	NDF				ADF			
	SS ¹	No SS ¹	SEM	P-value	SS ¹	No SS ¹	SEM	P-value
Diet	42.23 ^b	44.24 ^a	0.342	0.007	27.59 ^b	28.2 ^a	0.224	0.0541
Effluent	20.44 ^b	23.35 ^a	0.270	< 0.0001	13.56 ^b	14.47 ^a	0.184	< 0.0001
Digestibility ² , %	40.77 ^a	34.79 ^b	1.318	0.0012	39.51	36.18	1.393	0.0945

¹Fiber analysis was performed with (SS) or without (No SS) sodium sulfite during the neutral detergent fiber (NDF) procedure.

²True NDF or acid detergent fiber (ADF) digestibility in two replicated continuous culture experiments

^{ab} Means within effect (% NDF or %ADF) with no superscripts in common within the same row are statistically significant

Simulated foregut digestibility

Dietary treatment did not affect DM or OM digestibility ($P > 0.10$; Figure 3-3). Grape seed extract inclusion decreased CP digestibility ($P < .0001$) compared to Control. However, Mid diet inhibited enzymatic CP digestion to a greater extent than High diet ($P = 0.05$; Figure 3-3). Fiber digestibility was low (approximately 7-12%) and GSE inclusion affected NDF and ADF digestibility ($P \leq 0.002$), but no pattern is evident.

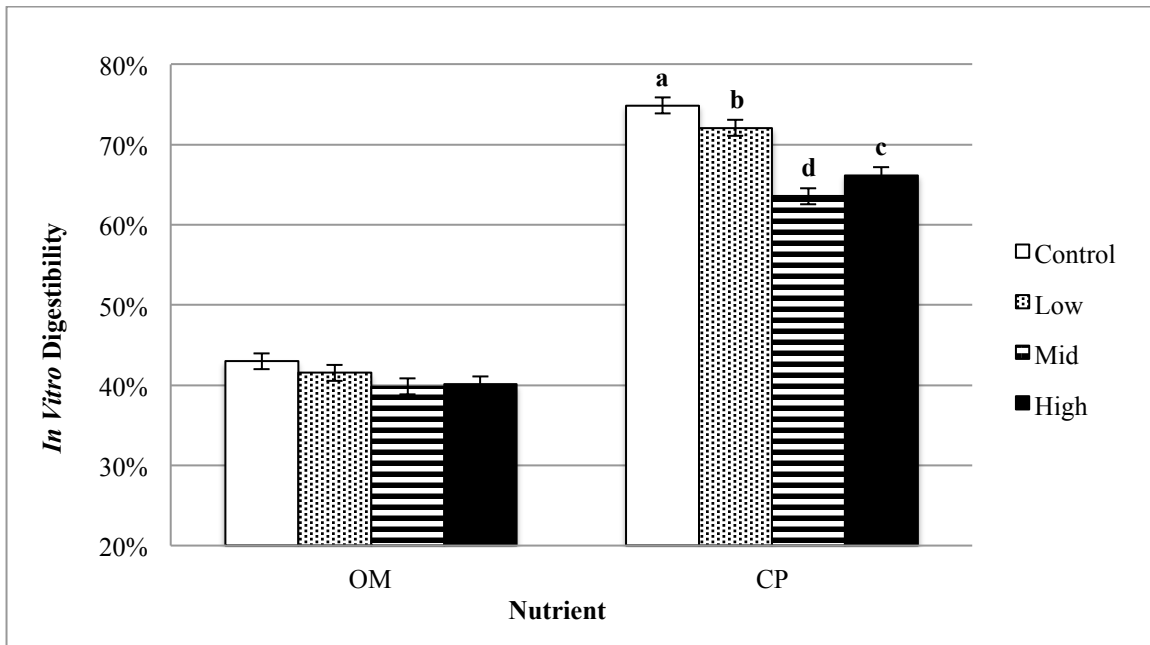


Figure 3-3. Continuous culture diet organic matter (OM) and crude protein (CP) *in vitro* gastric and pancreatic digestibility (%). Dietary treatments consisted of Control = basal continuous culture diet; Low = basal diet + 1.33% grape seed extract (GSE); Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE.

Continuous culture results

Effect of continuous culture run (i.e. replication) was tested for all variables. Unless otherwise discussed, there were no run by treatment or species interactions ($P > 0.05$), indicating that variables responded similarly in each experimental replication. Generally, if run effect was significant, values for all variables (nutrient digestibility (Table 3-7), pH, NH_3 and VFA concentrations (Table 3-9)) were slightly greater in the second continuous culture run.

Digestibility and microbial measurements

Nutrient digestibility and microbial measurements are presented in Table 3-6. There were no significant species by treatment interactions measured for these variables ($P \geq 0.10$). Grape seed extract inclusion quadratically affected OM and ADF digestibility ($P \leq 0.02$) and tended to decrease NDF digestibility ($P = 0.06$). Contrary to our hypothesis, crude protein digestibility was not affected ($P = 0.36$). Increasing GSE inclusion linearly increased ($P < 0.0001$) microbial OM production (OM_m , g/d) 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 (hour) and most interaction effects including hour were significant ($P < 0.0001$). This result was expected because pH, VFA and NH_3 concentrations are a function 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 ($P = 0.0012$). Both horse and rhino fermenter NH_3 concentrations decreased linearly with increasing GSE inclusion ($P \leq 0.05$), however species differences in the slope resulted in detection of a species by grape seed extract interaction. All horse fermenters fed Control diet had a spike in NH_3 concentration at 6 h after feeding (Figure 3-6), but no other species-treatment combinations demonstrated this same effect. Within treatment, horse fermenters had greater NH_3 concentration at every time point measured

(Figures 3-4, 3-5, and 3-6). Ammonia concentrations did not differ between runs ($P \geq 0.17$).

Overall, fermenter pH values did not vary extensively throughout the experiment (range: 6.06 – 7.04). Due to high replication and low variation within fermenters, a species by hour interaction was measured ($P < 0.0001$; Table 3-8). As an hour effect is expected, species main effect was considered. Horse fermenters consistently had greater pH than rhino fermenters (6.83 vs. 6.68, $P < 0.0001$). Dietary treatment did not affect pH values ($P = 0.73$).

A significant run by hour interaction was measured for total VFA concentrations ($P = 0.01$; Table 3-9). However, the main effect of run was not significant ($P = 0.23$). Typically, VFA concentrations peak in fermentation experiments between 2-4 post feeding. In run 1, total VFAs peaked (101.08 mM) at 4 hours post-feeding whereas the peak (102 mM) was measured at 2 h in run 2. This difference would not likely be biologically significant *in vivo* as there is generally a slow, continuous flow of feed to the cecum.

There was no interaction between species and treatment for VFA concentrations ($P \geq 0.10$). Therefore, main effects of treatment on VFA concentrations are presented in Table 14. Total VFAs, acetate, and butyrate concentrations did not differ between species. Rhino fermenters had greater propionate concentrations (22.85 vs. 19.86; $P = 0.002$), resulting in lower acetate:propionate ratios compared to horse fermenters ($P = 0.0008$). Fermenter species also affected isobutyrate, valerate, and isovalerate concentration ($P \leq 0.03$). Dietary treatment did not affect acetate, butyrate, or

acetate:propionate ratios ($P \geq 0.10$). Total VFA concentrations were lower in High treatment compared to Control ($P = 0.04$); and propionate, isobutyrate, valerate, and isovalerate concentrations decreased with GSE inclusion ($P \leq 0.02$).

Table 3-1. Effects of grape seed extract (GSE) treatment, species inoculum, and their interaction on continuous culture nutrient digestibility and microbial production measures.

Nutrient Digestibility ⁴ , %	Grape Seed Extract Treatment ¹ Effect						Species ² Effect				Interaction ³ Effect
	Control	Low	Mid	High	SEM	Treatment P-value	Rhino	Horse	SEM	Species P-value	Treatment x Species P- value
OM	46.55 ^b	42.46 ^b	48.36 ^b	55.85 ^a	0.0247	0.0028	46.95	49.66	1.553	0.2397	0.8908
CP	55.57	48.24	49.72	52.42	0.0338	0.3578	49.43	53.85	2.342	0.1764	0.6663
NDF	46.32	38.53	37.51	41.32	0.0273	0.0633	40.42	40.99	1.823	0.8208	0.2261
ADF	44.91 ^a	36.83 ^b	35.61 ^b	41.25 ^{ab}	0.0268	0.0367	39.66	39.10	1.765	0.8198	0.1484
Microbial Measures											
MOEFF ⁵	20.88	22.21	23.73	23.70	1.173	0.1602	23.76	21.68	0.794	0.0639	0.3423
OM _m , g/d	2.75 ^b	2.86 ^b	3.72 ^a	4.29 ^a	0.284	0.0004	3.66	3.17	0.190	0.0672	0.8610

¹ 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

² Fermenters were inoculated with fecal microbial populations from either black rhinoceros (Rhino) or domestic horses (Horse).

³ Experiment was designed as a 2x4 factorial to test the interaction of GSE treatment and species. No significant interactions were measured for any variables described here.

⁴ Organic matter (OM), crude protein (CP), and neutral and acid detergent fiber (NDF, ADF) true digestibility was measured.

⁵ Microbial efficiency (MOEFF); g microbial nitrogen outflow/ kg OM truly digested.

⁶ g of microbial organic matter produced per d

^{ab} Means within effect (treatment or species) with no superscripts in common within the same row are statistically significant

Table 3-2. Effect of continuous culture run (i.e. replication) on nutrient digestibility and microbial production measurements.

Run	Nutrient digestibility ¹ , %				Microbial production measurement	
	OM	CP	NDF	ADF	MOEFF ²	OM _m , g/d ³
1	45.99 ^b	48.81	40.20	36.12 ^b	18.88 ^b	3.04 ^b
2	50.79 ^a	54.17	41.64	43.19 ^a	26.34 ^a	3.76 ^a

¹ Continuous culture true nutrient digestibility (%): organic matter (OM); crude protein (CP); neutral and acid detergent fiber (NDF, ADF)

² Microbial efficiency (MOEFF); g microbial nitrogen outflow/ kg OM truly digested.

³ g of microbial organic matter produced per d

^{ab} Means with no superscripts in common within the same column are statistically significant

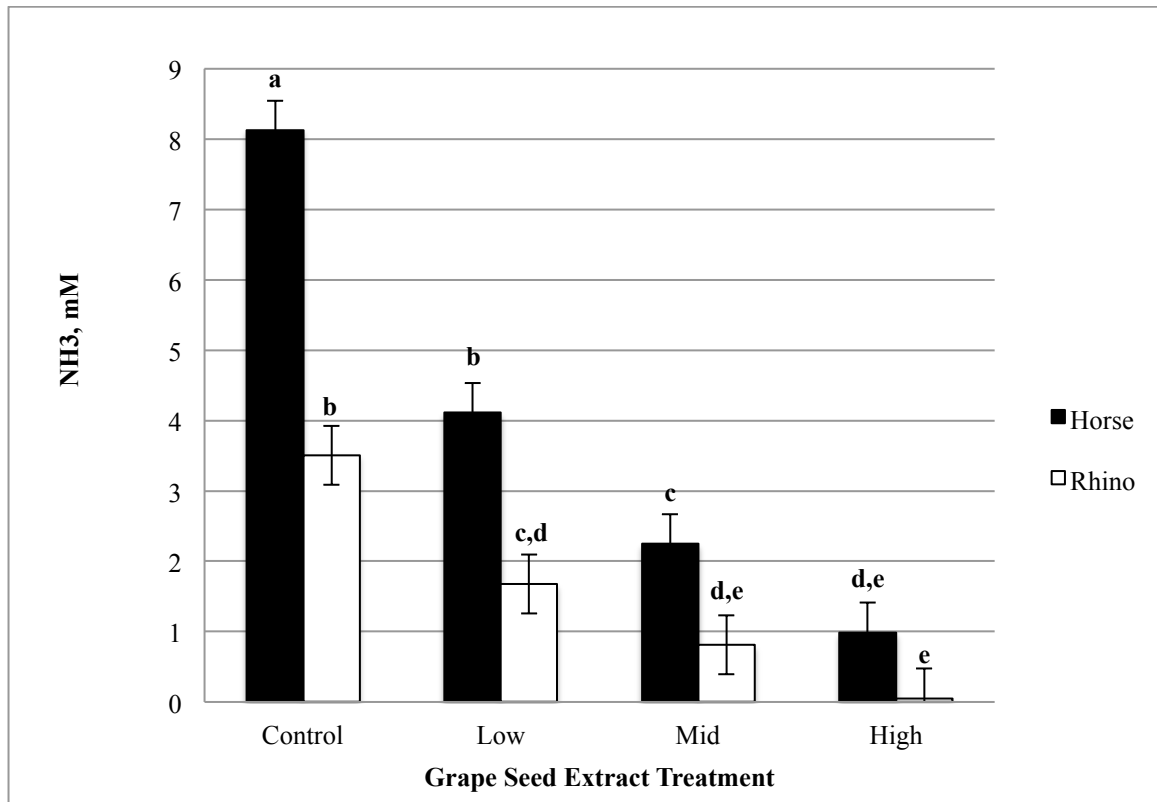


Figure 3-4. Effects of continuous culture dietary treatment and inoculum species on overall fermenter ammonia (NH_3) concentrations (mM). Species by treatment interaction $P = 0.0012$. Dietary treatments consisted of Control = basal continuous culture diet; Low = basal diet + 1.33% grape seed extract (GSE); Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE. Fermenters were inoculated with fecal microbial populations from either black rhinoceros (Rhino) or domestic horses (Horse)

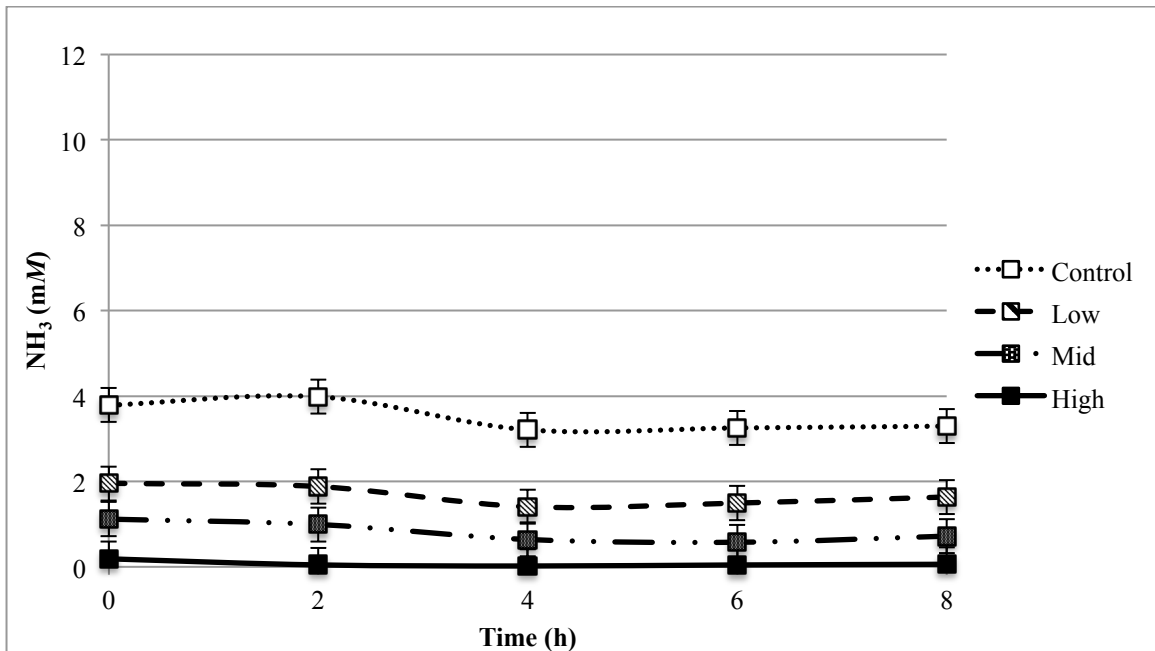


Figure 3-5. Ammonia (NH_3) concentration (mM) of black rhinoceros fecal inoculated continuous culture fermenters measured immediately prior to (0 h) and 2, 4, 6 and 8 h after feeding. Dietary treatments consisted of Control = basal continuous culture diet; Low = basal diet + 1.33% grape seed extract (GSE); Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE

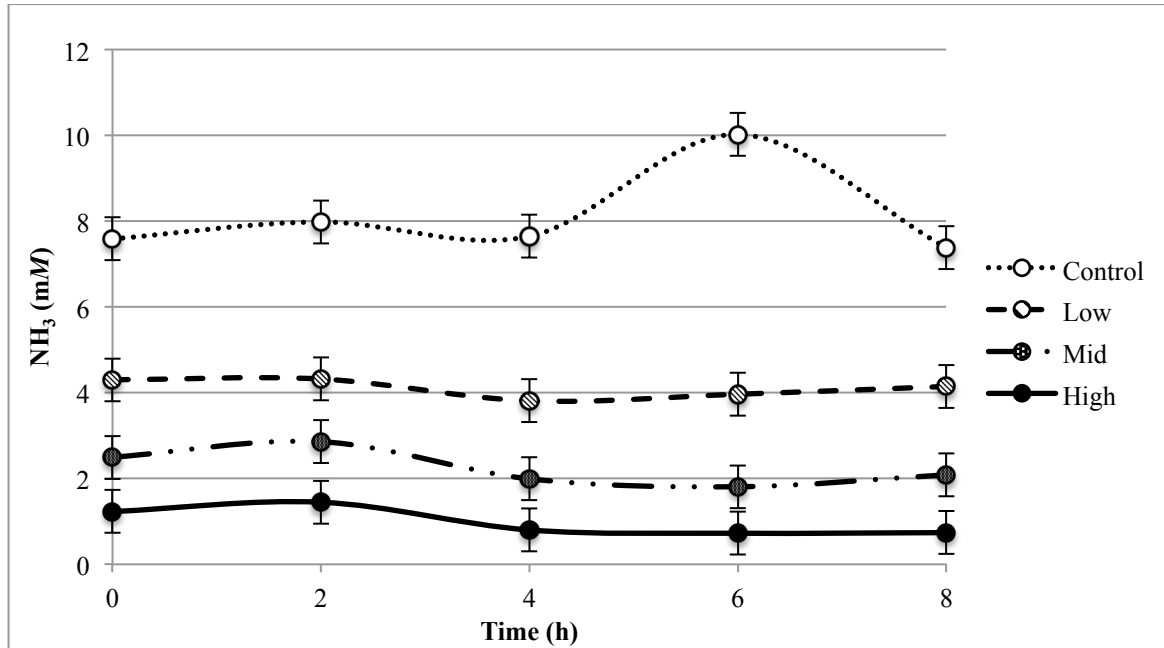


Figure 3-6. Ammonia (NH₃) concentration (mM) of domestic horse fecal inoculated continuous culture fermenters measured immediately prior to (0 h) and 2, 4, 6 and 8 h after feeding. Dietary treatments consisted of Control = basal continuous culture diet; Low = basal diet + 1.33% grape seed extract (GSE); Mid = basal diet + 2.66% GSE; High = basal diet + 4.00% GSE

Table 3-8. Effect of species, hour after feeding, and their interaction on continuous culture fermenter pH values

Species ²	Hour ¹					SEM	P-values		
	0	2	4	6	8		Species	Hour	Species x Hour
Rhino	6.83	6.58	6.59	6.68	6.73	0.019	< 0.0001	< 0.0001	< 0.0001
Horse	6.93	6.78	6.80	6.78	6.85	0.017			

¹Fermenter pH measurements were taken prior to (0 h) and 2, 4, 6, 8 h after the morning feeding.

²Continuous culture fermenters were inoculated with fecal microbial populations from either black rhinoceros (Rhino) or domestic horses (Horse).

Table 3-9. Effect of continuous culture run on fermenter total volatile fatty acid³ (VFA) concentration (mM) measured at multiple time points after feeding.

CC Run ²	Hour ¹					SEM	P-values		
	0	2	4	6	8		Run	Hour	Run x Hour
1	82.65	99.08	101.08	86.95	84.95	2.075	0.2432	<0.0001	0.0111
2	85.33	102.50	96.45	93.65	92.42	1.856			

¹Fermenter samples were taken prior to (0 h) and 2, 4, 6, 8 h after the morning feeding and measured for total VFA concentration (mM).

²Two replicated continuous culture experiments were conducted

³Total VFA = Acetate + Propionate + Butyrate + Isobutyrate + Valerate + Isovalerate

Table 3-10. Volatile fatty acid (VFA) concentration of continuous culture fermenters

Fermenter measurement ³	VFA	Species ¹				GSE Treatment ²					
		Horse	Rhino	SEM	<i>P</i> -value	Control	Low	Mid	High	SEM	<i>P</i> -value
Ace:Pro⁴		3.00 ^a	2.68 ^b	0.070	0.0008	2.70	2.89	2.95	2.86	0.108	0.2254
VFA⁵ concentrations, mM											
Total		90.74	94.57	2.075	0.4296	99.35 ^a	92.18 ^{a,b}	92.45 ^{a,b}	85.05 ^b	3.215	0.0430
Ace		59.22	60.55	1.417	0.8709	63.04	59.85	60.37	55.42	2.195	0.1083
Pro		19.86 ^b	22.85 ^a	0.524	0.0020	23.69 ^a	20.92 ^b	20.80 ^b	19.44 ^b	0.812	0.0161
But		8.01	7.53	0.297	0.3196	8.22	7.74	7.78	7.31	0.461	0.6609
Isobut		0.7 ^a	0.58 ^b	0.019	<.0001	0.79 ^a	0.67 ^b	0.61 ^b	0.48 ^c	0.030	<.0001
Val		2.38 ^b	2.7 ^a	0.083	0.0311	2.93 ^a	2.54 ^b	2.49 ^b	2.12 ^b	0.128	0.0047
Isoval		0.57 ^a	0.36 ^b	0.020	<.0001	0.68 ^a	0.48 ^b	0.40 ^b	0.28 ^c	0.030	<.0001

¹ Continuous culture fermenters were inoculated with fecal microbial populations from either black rhinoceros (Rhino) or domestic horses (Horse).

² 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.

³ Fermenter samples were taken every 2 h (up to 8 h) after the morning feeding. VFA measurements presented are averaged across all h.

⁴ Acetate:propionate ratio

⁵ VFAs measured: Total = Acetate (Ace) + Propionate (Pro) + Butyrate (But) + Isobutyrate (Isobut) + Valerate (Val) + Isovalerate (Isoval)

^{abc} Means within effect (species or treatment) with no superscripts in common within the same row are statistically significant

DISCUSSION

Tannin analysis

Three quantification and characterization assays were chosen to evaluate dietary tannin concentration. The acid butanol assay for proanthocyanidins (i.e. condensed tannins) has been applied in field research (Kipchumba, 2002; Helary et al., 2012), and iron-binding assays (Ward and Hunt, 2001) and protein precipitation assays (Ward et al., 2005) have been used to quantify black rhinoceros dietary tannin concentration in zoos. Comparisons across studies and methods must be made with caution. Inconsistent or inappropriate standards and method conditions complicate interpretation and comparison of direct results. Limitations of each assay are discussed in Chapter 2 of this thesis. In this context, the results of all three methods can be useful for general tannin characterization of the diets used in this study.

All three methods confirmed that linear tannin concentration increases as GSE inclusion increased. No GSE was added to the Control diet, yet low tannin concentrations were detected by all methods, indicating that the basal diet contains small amounts (approximately 0.3%) of polyphenols. Grape seed extract was assayed using the same methods and was estimated to contain 430 mg CT/g DM by the acid butanol assay and 640 mg CT/g DM by the PPP assay. Interestingly, the continuous culture diets demonstrated an opposite trend, with greater CT concentrations determined by the acid butanol assay rather than PPP. Results from the iron-binding capacity assay indicated greater proportions of tannic acid equivalents as GSE inclusion decreased. Furthermore, anthocyanidin monomer analysis indicated greater proportions of delphinidin in purified diet CT as compared to purified GSE. These findings suggest that tannins inherent in the

basal diet differ from those in GSE and may be small polyphenolic molecules lacking strong protein binding ability (Harbertson et al., 2014) but still efficiently bind iron (Mladěnka et al., 2011). Greater (+)-catechin equivalents of Control diet than CT concentration (determined by the acid butanol assay) suggests these molecules may not be small proanthocyanidins or monomeric anthocyanins, but other polyphenolic molecules that do not result in color formation when reacted with butanol HCl.

Protein precipitation capacity of the continuous culture diets is almost completely derived from GSE addition. Grape seed extract contains 636 mg CT/g and when supplemented at 4% of diet DM the High diet is calculated to contain about 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 match predicted CT concentrations.

Microbial metabolism of polyphenolic compounds may convert large polymerized tannins into smaller, bioactive compounds that influence host health (Kemperman et al., 2010). A lack of effluent CT detection by the PPP method suggests that hindgut microflora modified or depolymerized GSE and dietary condensed tannins in all experimental diets. Degree of CT polymerization is positively correlated with its ability to precipitate protein (Harbertson et al., 2014). Therefore, results indicate that both horse and rhinoceros microflora depolymerized CT in this experiment to a size that was too small to effectively bind protein. Microbial metabolism is a crucial factor affecting polyphenol bioavailability and recent interest in the gut microbiome offers new potential to understand the variability of reported polyphenolic bioactivities based on variable gut

microbial populations (Aura, 2008; Gross et al., 2010; Dall'Asta et al., 2012; Margalef et al., 2014).

Condensed tannin concentrations tested in this research reflect typical CT content in the natural diets of black rhinoceros. Helary et al. (2012) analyzed 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 – 4.3% of DM. Other studies report CT concentration ranges from 0.2-18% 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 sulfite on fiber analysis of tannin containing samples

The ANKOMTM detergent fiber analysis system is routinely used for fiber determination in ruminant nutrition laboratories. This procedure recommends adding heat-stable α -amylase and sodium sulfite to the neutral detergent solution for improved assay accuracy. However, the use of SS in NDF determination of high tannin samples has been questioned (Gomes et al., 2012) and results appear to depend on tannin type and concentration (Pagán et al., 2009). Terrill et al. (1994) reported that both NDF and ADF variation in forage samples were minimized when SS was used with crucible fiber determination methods. Similar results suggested SS inclusion and sequential ADF determination should be used for tannin-containing samples with the ANKOMTM analysis system (Terrill et al., 2010). In this study, forage based diet samples with moderate levels

of CT from GSE were more accurately analyzed for fiber components with addition of sodium sulfite for NDF analysis followed by sequential ADF analysis.

Simulated foregut digestibility

An *in vitro* enzymatic digestion procedure was attempted to mimic the feed form presented to cecal microbial populations *in vivo*. Although this procedure is not feasible for application with continuous culture experiments, results may predict GSE effects on foregut digestibility *in vivo*. As expected, enzymatic fiber digestibility was low (7-12%) meaning the majority of fibrous feed components would be fermented hindgut microbial populations. Grape seed extract inclusion decreased enzymatic CP digestibility compared to control (67% vs. 75%) and the effect was most pronounced in Mid diet (64%) followed by High (66%). Reduced apparent CP digestibility due to tannin intake is documented in monogastric animals (Robbins et al., 1991; Wren et al., 2013) but depends on CT source and concentration (Clauss, 2003). Other studies report no CT effect on CP apparent digestibility (Hagerman et al., 1992). Black rhinoceros diets in captivity are typically comprised of a legume/grass hay mix and a pelleted ingredient resulting in high CP content (15-20%; Clauss et al., 2012). In comparison, free-ranging black rhinoceros diets are lower in protein (6-15%; Kipchumba, 2002; Clauss and Hatt, 2006) suggesting that a small decrease in CP digestibility would not likely result in protein deficiency in captive black rhinoceros populations. In agreement with this hypothesis, Clauss et al. (2007a) reported no influence of tannin supplementation (0.5-1.5% of diet DM as quebracho tannins or tannic acid) on true protein digestibility.

Continuous culture

Contrary to our hypothesis, the results support using 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 indicates that hindgut fermentation in both species responds similarly to changes in dietary GSE CT concentrations. Interpretation of these results is limited to hindgut digestion and assumes that similar nutrients are reaching the hindgut in both species, which has not yet been evaluated *in vivo*. Comparisons of digestion coefficients between black rhinoceros and other grazing species found shorter particle retention times and decreased fiber digestibility in the browsing rhinoceros species (Clauss et al., 2006a; Steuer et al., 2010). Clauss et al. (2006a) measured total tract digestion coefficients from 8 black rhinoceros on 3 diets and compared their data to horse digestion coefficients from multiple studies using similar diets. These researchers reported lower OM and crude fiber digestion coefficients in the black rhinoceros. This study found no species effect on nutrient digestibility by hindgut microbial populations. Reduced OM and fiber digestibility may have been due to faster particle passage rates 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 supports this hypothesis. However, lower acetate:propionate ratios, NH₃ concentrations, pH, and greater MOEFF and OM_m in rhinoceros fermenters indicated that the type of fermentation and microbiome composition may differ. Fecal and inoculum samples from each species were saved for metagenomic analysis to provide further insight, but this has yet to be completed.

Increasing GSE inclusion appeared to stimulate microbial growth, as indicated by a linear increase in microbial OM production, decreased NH₃ 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 NH₃ concentrations. Nitrogen released from dietary protein is stoichiometrically accounted for as either NH₃-N or microbial protein. Therefore, it appears that dietary N exceeded microbial requirements for the growth level obtained at a 3.8% h⁻¹ dilution rate (Meng et al., 2000; Brooks et al., 2012). This hypothesis is supported by a lack of GSE effect on microbial efficiency, 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 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 cecal microbial biodiversity (Viveros et al., 2011). It would be interesting to compare microbial population composition among GSE treatment levels to see if the stimulatory growth effect was universal or specific to microbial taxonomic and/or functional groups.

CONCLUSION

Domestic horse and black rhinoceros hindgut microflora nutrient digestibility and fermentation responses to GSE did not differ. Results of 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 are 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 nutrient digestibility or microbial viability and fermentation. *In vivo* trials are needed to determine supplementation levels necessary to limit iron bioavailability.

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APPENDICES

APPENDIX A - TANNIN ANALYSIS METHODS

Condensed Tannin Extraction Procedure

Adapted from methods by Strumeyer and Malin (1975) and Naumann et al. (2013)

Supplies Needed

- Stir Plate(s)
- 600 mL beakers (or large enough to hold sample and solvent)
- Side arm flasks
- Analytical balance
- Smaller beakers to use for pouring solvent
- Buchner funnels – one fitted with a stopper and 1-way valve in the small opening
- Separatory funnel and ring stand to hold it up
- Filter paper
- Sephadex-LH 20
- 70:30 (vol/vol) acetone:water
- 50:50 (vol/vol) methanol:water
- Diethyl Ether
- Glass stir rods
- Nalgene containers to lyophilize samples in

Notes

Throughout the procedure, make sure to keep the flask with the sample in it appropriately labeled. The sample is transferred to many different containers/flasks throughout the process so it is easy to lose track of which sample is in which container.

This is a long procedure and there are many potential stopping points throughout (Denoted with (*)) following the last step). After these steps the sample can be kept in the flask, covered, and labeled with the sample ID, the solvent it is currently in, and which step is to be resumed next time.

Procedure

1. Weigh 10-15 g of sample into 600 mL beaker
2. Place on stir plate with a stir bar and add 250 mL of acetone water/10 g of sample
3. Stir for 30 min
 - a. During this time, mix up the 50% MeOH to allow sufficient time for the mixture to cool (reaction is exothermic)
4. After sample is solubilized, pour sample and acetone mixture through a Buchner funnel fitted with filter paper
 - a. Material remaining on the filter paper can be disposed

5. Transfer the filtered solution to a separatory funnel and place in the ring stand under a chemical hood
6. Estimate the volume of solution in the separatory funnel, and add an equal volume of diethyl ether.
7. Close with stopper (hold it in place tightly) and shake to mix for 30-45 seconds
8. Allow the two layers to separate for at least 5 minutes or until 2 distinct layers have formed to completion
9. Recover the bottom aqueous layer in a flask and dispose the ether solvent layer (the top layer)
10. Repeat steps 5-9 at least 3 times or until the upper ether layer runs cleaner and maintains a consistent clarity.
11. After last separation, and before collecting the final sample, rinse the collection flask with DI water to remove any impurities from the last washes
12. Collect the final aqueous layer in the one-arm flask
13. Remove flask from the hood and close with a stopper fitted with a long straw through the hole.

14. Connect a vacuum tube and pull a vacuum strong enough to cause slight bubbling, but not splashing of the liquid in the bottom of the flask.
 - a. This step helps evaporate acetone solvent
15. Continue flushing with air/evaporation until there is no longer a detectable scent of acetone, methanol or ether – usually takes ~1 h *

16. Set up another side arm flask with a Buchner funnel and attached stopper.
 - a. Place appropriate sized filter paper in the funnel and secure it by adding a small volume of DI water
17. Filter the sample solution through the funnel by pulling a weak vacuum (sample can be slightly foamy so a weak vacuum helps reduce the foam)
 - a. You can wash the old flask with a small volume of methanol water to recover as much sample as possible.
 - i. This can also help reduce the amount of foam
 - b. Filter this solution as many times as necessary to clean the sample and get rid of any large feed particles or chunks *

18. Set up the large Buchner funnel by closing the bottom end with a #2 stopper attached to the 1 way-valve connected to the 4-way stopcock.
19. Hook up a large sidearm flask to the vacuum and fit the funnel with filter paper.
 - a. Add a small volume of DI water to secure paper to funnel

If using NEW Sephadex:

20. Close valve and add ~ 40-50 g new, dry powdered Sephadex to the funnel.
21. Add enough MeOH to create a thick slurry
 - a. Subjective, actual volume is not crucial

If reusing already hydrated (in MeOH) Sephadex:

- Used Sephadex should still be a clean white color. It is good to wash with acetone prior to use even if it is already a bright white color.
20. Using a glass rod, mix Sephadex in bottle well until a uniform mixture is achieved. Close valve on funnel.
 21. Pour in enough Sephadex to fill a little less than half of the funnel.

 22. Stir Sephadex using a glass rod until a thick, uniform slurry is achieved.
 - a. be very careful to not move the filter paper which will cause Sephadex granules to fall through
 23. Pour in sample solution.
 24. Stir with a glass rod for ~ 5 minutes to mix sample well into Sephadex.
 25. Let sit for ≥ 5 min to allow condensed tannins to bind to the Sephadex particles.
 - a. The sample is now bound to and contained in the Sephadex column so the liquid collected is waste
 26. Open valve (be careful to not spill sample out of funnel) and turn on vacuum slowly until most of the liquid has drained out and the Sephadex column appears dry and may start cracking.
 - a. Pay attention to the color of the liquid layer, which should lighten and clear up with each washing.
 27. Wash sample with methanol water and stir.
 - a. If liquid does not start running out immediately after adding in MeOH you can leave the valve open during this time.
 28. Turn vacuum back on slowly and allow liquid to drain until the column is dry again.
 - a. Discard the liquid down the drain and pay attention to color changes.
 - b. If fine Sephadex particles are observed, do not discard, re filter the liquid through the Sephadex column until the liquid layer is clear again.
 29. Repeat steps 26 – 28 ~ 10 times or until the collected liquid is running a clearer and is a lighter color (may still have a yellow or brown tint)
 30. Discard final liquid collection and wash the flask with DI water.

 31. Close the valve. Fill the column with enough acetone water to create a slurry
 - a. Will feel thinner than it did with methanol
 32. Mix in well and be extra careful to not disturb the filter paper
 33. Let sit for ≥ 10 min. This step dissociates the CTs from the Sephadex particles.

- a. The sample is now solubilized in the acetone layer, **DO NOT dispose of the liquid layer**
34. Open valve and slowly turn on the vacuum to collect the CTs solubilized in acetone.
35. Repeat steps 31 – 34 ~ 2 more times until the Sephadex returns to the original white color.
 - a. The more acetone added at this step increases the amount of time it will take to evaporate off during the final steps of the procedure. *
36. Set up another side arm flask with a Buchner funnel and filter paper.
37. Filter the collected liquid until it is clean of any Sephadex particles or clumps of sample.
 - a. Recover any Sephadex particles.*
38. Remove the Buchner funnel and plug the flask with a stopper fitted with a long straw and again pull a slow vacuum
 - a. Strong enough to cause bubbling or ripples in the liquid but no splashing
39. Evaporate for ~1-2 h or as long as it takes for the acetone/solvent smell to be non-detectable. *
40. During the evaporation step, wash the remaining Sephadex with acetone ~2 times until clean and white.
 - a. Filter the liquid through a new filter paper in a different flask if necessary to remove any Sephadex that escaped.
41. Collect the Sephadex from the funnel and filter paper in a beaker and rehydrate with Methanol/water to and stir to create a pourable slurry. Return the mixture to the original bottle and store at 4°C.
42. After sample solution no long has a detectable scent of solvent/acetone collect the liquid into an appropriately sized Nalgene bottle, cap, and store in a -80C freezer until ready to freeze-dry.

Acid Butanol Assay of Extractable and Unextractable Proanthocyanidins

Based on methods by Ann Hagerman, 2011, Tannin Handbook

Supplies Needed:

- 2 ml microfuge tubes
- 15 ml centrifuge tubes
- 50 ml centrifuge tubes
- Rotary tube extractor
- Boiling water bath
- Microcentrifuge
- Centrifuge
- Vortex
- 1 ml Pipets and tips
- Repeater pipets
- Serological pipet aid and 50 ml serological pipets
- Spectrophotometer set at 550 nm

Reagents:

- Acid butanol
 - o Mix 950 ml of n-butanol with 50 ml concentrated HCl
- 2% Ferric Ammonium Sulfate in 2 N HCl
 - o Using a 100 ml volumetric flask, bring 16.6 ml of concentrated HCl up to 100 ml with DI water to make 2 N HCl.
 - o Dissolve 0.5 g $\text{FeNH}_4(\text{SO}_4)_2 \times 12 \text{H}_2\text{O}$ in 25 ml of 2 N HCl.
 - o Store in a dark bottle.
- Reagent grade methanol

Extractable Proanthocyanidins

Preparation:

- Heat water bath to 98°C. Find a rack that can be placed in the water bath to hold tubes.
- Label 1 set of 2 ml microtubes and 3 sets of 15 ml tubes

Procedure:

1. Weigh 200mg of dried and ground sample, in triplicate into a 2.0 flat bottom microfuge tube.
2. Add 2 mL of 70% Acetone/30% water to each sample, cap and vortex.
3. Place the tubes on a rotary extractor for 30 minutes.
4. Centrifuge the samples for 5 minutes at 13,000 x g, 20°C.
5. Remove 1 mL of supernatant and place it in a new labeled 15 ml centrifuge tube.
6. Aspirate and dispose of all remaining supernatant on the sample.

7. Repeat steps 3-5 two more times for a total of 3 extractions. Each extract is placed in a separate tube so that the % of total extractable material at each step can be determined.
8. Prepare a standard curve by pipetting 1000 μL of the 0%, 1%, 5%, 10%, and 20% stock standard CT solutions into 15 ml tubes. (Preparation of stock standard solutions described below)
9. Add 300 μL of methanol, 1600 μL of acid butanol, and 66 μL of ferric ammonium sulfate to each sample, standard, and to a new empty tube to create a blank.
10. Vortex. Then place all samples in a boiling water bath for 20 minutes.
11. Vortex. Replace in water bath for 25 more minutes. Set up the spec at this time (550 nm)
12. Read the absorbance of the standards and all samples at a wavelength of 550 nm against the methanol, acid butanol and ferric ammonium sulfate blank.

Unextractable Proanthocyanidins:

Preparation:

- Heat water bath to 98°C. Find a rack that can be placed in the water bath to hold 50 ml centrifuge tubes.
- Label 1 set of 50 ml centrifuge tubes.

Procedure:

1. Weigh 50 mg of each sample into a 50 ml centrifuge tube.
2. Add 5 ml of 70% acetone/30% water to each sample and cap and vortex before placing the tubes on a rotary extractor for 30 minutes.
3. Centrifuge samples for 5 minutes at 1800 rpm.
4. Remove and discard the supernatant.
5. Repeat steps 2-4 two more times.
6. After the third extraction, add 45 ml of acid butanol using a 50 ml serological pipet and 1.8 ml of ferric ammonium sulfate using a repeater pipet to the pellet in the centrifuge tubes.
7. Prepare a standard curve by pipetting 2.5 ml of the 0%, 1%, 5%, 10%, and 20% stock standard CT solutions into 15 ml centrifuge tubes. (Preparation of stock standard solutions described below)
 - a. Add 4 mL acid butanol and 16 μL ferric ammonium sulfate.
8. Create a blank by adding 4 ml acid butanol and 16 μL ferric ammonium sulfate to a 15 ml centrifuge tube.
9. Vortex all samples and standards to mix well.
10. Place the tubes in the boiling water bath for 20 minutes.

11. Vortex, then replace in the water bath for another 25 minutes. During this time, set up the spec (550nm)
12. Remove from water bath and centrifuge at 1,000 rpm for 5 minutes at 20°C.
13. Read the absorbance of the standards and all samples at a wavelength of 550 nm against the methanol, acid butanol and ferric ammonium sulfate blank.

Condensed Tannin Standards Preparation:

- Make a 20% stock solution of the appropriate purified condensed tannin source by dissolving 40 mg in 20 ml of 70% acetone 30% water. (This is the 20% standard)
- Use the 20% stock solution to make the following serial dilutions:
 - o 15% - Mix 2.25 mL of 20% solution with 750 μ L of acetone/water
 - o 12% - Mix 1.8 mL of 20% solution with 1.2 mL of acetone/water
 - o 10% - Mix 5.5 mL of 20% solution with 5.5 mL of acetone/water
 - o 7.5% - Mix 3 ml of 10% solution with 1 mL of acetone/water
 - o 5% - Mix 4 ml of 10% solution with 4 mL of acetone/water
 - o 2.5% - Mix 2 ml of 5% solution with 2 mL of acetone/water
 - o 1% - Mix 800 μ L of 5% solution with 3.2 mL of acetone/water
 - o 0% - 70% acetone 30% water

Calculations:

- Absorbance values are converted to μ g/ μ L CT standard equivalents using a standard curve generated under the same conditions (this can be calculated by the spec if the standard curve is read prior to the samples).
 - o The concentration is then adjusted for all dilution steps so that the total μ g from the 50 mg sample can be calculated.
- For the extractable tannins, the % of total tannin recovered at each step is calculated.
- The sum of extractable and unextractable tannins is used to calculate total condensed tannin in the samples.

Protein Precipitable Phenolics

Adapted from: Hagerman and Butler, 1978, J. Agric. Food Chem. 26, 809-812

Notes:

- Weigh out samples into 3 tubes to create triplicates.
 - Each tube will then be duplicated within the assay.
- Include a control in each assay.

Supplies needed:

- Analytical balance
- 2 ml flat-bottom microcentrifuge tubes
- 1.5 ml conical microcentrifuge tubes
- Microcentrifuge tube racks
- Repeater Pipets and tips
- 5 beakers to hold reagents during assay
- Side arm flask fitted with vacuum hose and stopper
- Gel-loading (or any small tipped) pipet

Reagents

- **Buffer A** (0.20 M Acetic acid, 0.17 M NaCl, adjusted to pH 4.9):
 - Prepare the following two solutions to make large volumes of Buffer A:
 - 2 M acetic acid, 1.7 M NaCl
(Store at 4°C)
 - Add 114 ml glacial acetic acid to about 800 ml DI water in a 1 L volumetric flask
 - Add 99.4 g NaCl
 - Swirl to mix and dissolve NaCl – add more water if necessary
 - Bring to volume (1 L) with DI H₂O
 - 2 M sodium acetate, 1.7 M NaCl
(Store at 4°C)
 - Add 164.1 g sodium acetate anhydrous to about 800 ml DI water in a 1 L volumetric flask
 - Add 99.4 g NaCl
 - Swirl to mix and dissolve NaCl – add more water if necessary
 - Bring to volume (1 L) with DI H₂O
 - Mix 40 ml of acetic acid solution with 60 ml of sodium acetate solution in a 1 L volumetric flask
 - Bring to volume (1 L) with DI H₂O
 - Check the pH, it should be 4.9

- Adjust if necessary using 1 M NaOH
 - Store at 4°C

- **Bovine Serum Albumin (BSA) solution:**
(5 mg BSA / ml buffer A)
 - Weigh 250 mg BSA (Sigma A-6003) and solubilize in 50 ml of buffer A
 - Mix well
 - Store at 4°C

- **SDS/triethanolamine solution:**
(1% SDS, 5% TEA)
 - Weigh 10 g Sodium dodecyl sulfate (**SDS**, electrophoresis grade) into about 800 ml of DI H₂O in a 1 L or larger beaker with a stir bar
 - Gently mix on a stir plate (SDS is a detergent and will foam easily)
 - While mixing, add 50 ml triethanolamine (**TEA**, C₆H₁₅NO₃ ≥ 98%)
 - Triethanolamine becomes yellow with age. You can use it when it is light yellow but if it becomes brown you should discard and purchase a new bottle.
 - Slowly pour mixture into a 1 L volumetric flask and bring up to solution with DI H₂O
 - Store at 4°C

- **Ferric Chloride Solution** (0.01 M FeCl₃ in 0.01 M HCl):
 - Make 0.01 M HCl by diluting 0.83 ml concentrated HCl (12.1 N) in 1 L DI H₂O. Mix well.
 - Weigh 1.62 g ferric chloride (FeCl₃, very hygroscopic – be careful not to spill any) and add to a 1 L volumetric flask containing about 800 ml of DI H₂O
 - Swirl to mix and dissolve FeCl₃
 - Bring to volume using DI H₂O
 - Transfer to a 1 L beaker and allow to sit for several hours
 - Gravity filter solution through a Whatman #1 filter paper to remove particles
 - This is a very slow process
 - Store at 4°C

Crude Extraction:

*Extract samples the same day of use

1. Weigh 50 milligrams of sample into a 2 ml flat-bottom microcentrifuge tube.
2. Add 1 ml of 50/50 methanol water
3. Invert tube until sample is no longer stuck at the bottom
4. Vortex for about 5 seconds
5. Lay tubes horizontally on a shaker table at speed 9, for 30 minutes
6. Centrifuge for 5 min at 13,000 RPM (highest speed)
7. Use the supernatant in the assay

Protein Precipitable Phenolics Assay

1. Label 1.5 ml conical microcentrifuge tubes
 - a. Remember to create duplicates for each sample extract tube
2. Add to each new tube:
 - a. 250 μ l buffer A
 - b. 50 μ l BSA solution
 - c. 50 μ l 50% methanol water
 - d. 50 μ l supernatant of sample extract
3. Vortex tubes for about 7 seconds and allow to incubate at room temperature for 30 minutes
4. Centrifuge for 5 minutes at 13,000 rpm
5. Using a gel loading pipet (very small tip) attached to a side arm flask and vacuum tube, Aspirate the supernatant
6. Wash the pellet by adding 250 μ l of buffer A
7. Vortex for about 5 seconds
8. Centrifuge for 5 minutes at 13,000 rpm
9. Aspirate off the supernatant
10. Dissolve the pellet by adding 800 μ l of SDS/TEA solution
11. Vortex vigorously until the pellets are completely dissolved
12. Quickly revortex each sample after the whole set is dissolved
13. Add 200 μ l of FeCl₃ solution to each tube
 - Create a blank sample by adding 800 μ l of SDS/TEA solution and 200 μ l of FeCl₃ solution
14. Vortex shortly
15. Incubate at room temperature for 30 minutes
 - a. During this time turn on the Spec and set up a fixed scan program to read at 510nm
16. Using a 1 ml capacity reusable cuvette, read the absorbance at 510 nm
 - a. Make sure to blank or zero the spec first

Standards preparation

1. Using purified condensed tannins (CT), prepare a CT solution (1 mg purified CT/ 1 ml DI H₂O)
 - a. Weigh 10 mg of purified CT material and dissolve in 10 ml of DI H₂O
 - b. mix well
2. Follow the protein precipitable phenolics assay using the CT solution in place of the extract
3. Use the following concentrations as a good starting point to create a standard curve:
 - a. You will have to adjust the concentrations to fit your sample absorbance readings if outside this range.

Buffer A	BSA	H₂O	μl CT solution
250 μl	50 μl	0 μl	700 μl
250	50	200	500
250	50	400	300
250	50	600	100
250	50	650	50

Determination of Iron-Binding Phenolic Capacity

Method based on Brune et al. (1991)

Supplies Needed:

- 50 ml centrifuge tubes
- analytical balance
- Boiling water bath
- (+)-Catechin hydrate
- Tannic Acid
- Spectrophotometer

Reagents:

- 1% acetate buffer:
 - o Mix 630 ml of 0.1 N acetic acid (5.75 ml glacial acetic acid/L) and 370 ml of 0.1 N sodium acetate (13.6 g sodium acetate trihydrate/L)
 - o Check pH, should be 4.4
- Dimethylformamide (**DMF**)-acetate solution:
 - o Mix equal volumes of DMF and the 1% acetate buffer
 - o Allow solution to cool to room temperature before use
- Gum arabic solution:
 - o 1g gum Arabic / 100 ml DI water
- Ferric ammonium Sulfate (**FAS**) Solution:
 - o 5 g FAS / 100ml of 1 M HCl
- FAS reagent:
 - o Mix 89 parts 1% acetate buffer, 10 parts gum arabic solution, and 1 part FAS solution
- Food Blank Reagent:
 - o Mix 89 parts 1% acetate buffer, 10 parts gum arabic solution, and 1 part 1 M HCl

Standards:

1. Create standard stock solutions (**400 µg/ml**) by dissolving 20 mg of purified catechin-hydrate or tannic acid in 50 ml of DMF-acetate solution. Vortex to mix.
2. **300 µg/ml**: Mix 15 ml of the 400 µg/ml stock solution with 5 ml DMF-acetate solution. Vortex to mix.

3. **200 µg/ml**: Mix 10 ml of the 400 µg/ml stock solution with 10 ml DMF-acetate solution. Vortex to mix.
4. **100 µg/ml**: Mix 10 ml of the 200 µg/ml solution with 10 ml DMF-acetate solution. Vortex to mix.
5. **50 µg/ml**: Mix 10 ml of the 100 µg/ml solution with 10 ml DMF-acetate solution. Vortex to mix.

Sample Extraction and Analysis:

1. Weigh 0.5 g of each sample (in duplicate) into a 50 ml centrifuge tube, record weight.
2. Add 50 ml of DMF-acetate solution to each flask and cover with parafilm.
3. Wrap the flasks in foil to block light and mix for 16 hours on a shaker plate at room temperature.
4. After 16 h, centrifuge the tubes at 3500 x g for 15 min at 22°C
5. Pipet 2 ml of supernatant or standard solution into 2 10 ml test tubes.
6. Add 8 ml of the **FAS reagent** to one set of tubes (**set 1**) and 8 ml of the **food blank reagent** to the second set of tubes (**set 2**).
7. Create duplicate reagent blanks by mixing 2 ml DMF-acetate solution with 8 ml FAS reagent in a 10 ml test tube.
8. Create duplicate food blanks by mixing mixing 2 ml DMF-acetate solution with 8 ml food blank reagent in a 10 ml test tube.
9. Wait 15 min before reading the samples on the spec.
10. Read the absorbance of each **set 1** standard and sample at 578 nm and 680 nm versus the **reagent blank**.
11. Read the absorbance of each **set 2** standard and sample at 578 nm and 680 nm versus the **food blank**.

Calculations:

- Sample absorbance = Absorbance_{Set1} – Absorbance_{Set2}
 - o This is done at both 578 nm and 680 nm
- The absorbance spectra of the two kinds of Fe-phenolic complexes will overlap.
 - o The content of galloyl and catechol groups are calculated by using the linear regression equation from the standard curves of tannic acid and catechin-hydrate at the two respective wavelengths.

APPENDIX B – CONTINUOUS CULTURE PROTOCOLS AND PROCEDURES

Fecal Collection Protocol

- Collect entire feces as soon after excretion as possible.
- Record time since excretion, if known, or best estimate.
- Remove the outer layer to prevent bedding, soil or feed contamination

Materials and Equipment needed:

- CO₂ tank w/ regulator and hose
- Dry Ice
- Clean bucket / container to collect feces in
- Latex Gloves and Gloves to handle dry ice with
- Cooler filled with ice
- Storage containers (dry ice safe)
- Sharpies, pens, paper and labeling tape
- 1:3 glycerol/McDougall's buffer solution
- Scale
- Graduated Cylinders of multiple volumes
- Calculator
- Ziploc bags for DM sample

Procedure:

- a. After removing the outer layer and any contamination, take a representative sample for DM and nutrient determination
- b. Weigh the remaining feces, and record weight
- c. Purge the storage containers with CO₂ for 3 minutes
- d. Break up any clumps and mix the feces by hand.
- e. Add the cold solution of glycerol-McDougall's Buffer to the blender at 20% (vol/wt) of the weight of the feces in the blender
- f. Mix the buffer as evenly into the feces as possible.
- g. Add the mixture of feces and buffer to the storage containers and flush with CO₂ for 1 minute.
 - i. Keep the samples as cold as possible / over ice during this time.
- h. Label the containers and place in cooler with dry ice
- i. Store samples in -20°C freezer until use.

Fecal Inoculum Preparation

Based on methods by Sunvold et al., (1995) and Luchini et al. (1996)

Thaw Procedure:

1. 8 hours before starting the continuous culture remove feces from the -20°C freezer.
 - a. Remove only feces needed for 1 experiment at a time and leave the remainder completely frozen, make sure to use equal representation of each animal / species.
 - b. 1 kg / individual / species
2. Thaw frozen feces rapidly in a 37°C water bath.
 - a. Stir/swirl periodically throughout thawing process.
3. While thawing, prepare the pre-incubation media and warm the solution to 37°C.

*Always keep the rhinoceros and horse feces well separated to avoid microbial contamination.

Pre-Incubation Protocol:

1. While feces are thawing, prepare the pre-incubation media as described below.
 - *Some solutions can be prepared days ahead of time and some within a few hours of use.
 2. After the feces are thawed, using sterile tools, take one sample from each animal and place in a sterile, sealable container for future bacterial population analysis, immediately store in -80°C freezer.
 3. Weigh out exactly 1 kg feces / individual / species.
 - a. While weighing, continually flush with CO₂ and maintain an anaerobic environment.
 4. Flush the blender with CO₂ for 2 min.
 5. In the pan the feces were weighed in, combine the feces and 1 L of pre-incubation media and mix partially by hand to help break up and clumps and incorporate the media.
 6. Transfer the mixture into the blender and blend on low for 30 seconds to obtain a liquid consistency.
- Between animals of the same species the blender only needs to be rinsed with DI water, but it must be cleaned thoroughly and rinsed 3x with sterilized DI water between rhinoceros and horse samples. *

7. Combine all 3 animals / species in an autoclaved 4 L flask and flush with CO₂. Using a sterile stir rod, mix the samples on a stir plate for ~10 min, on a low heat setting (~ 2). Cover with Parafilm.
8. Place the flasks in a 37°C water bath for 6 hours prior to inoculating the continuous culture.
 - a. Hourly, swirl the flasks to mix contents.

Preparation of media:

1 L of pre-incubation media is needed per 1 kg of feces

Pre-warm the media to 37°C prior to mixing with feces

▪ Equipment needed

- Scale
- Autoclave
- CO₂ gas
- Graduated Cylinders
- Sterile Beakers and Pyrex jars
- 5, 10, & 25 mL serological pipet tips
- pipet aid
- pipet
- 200 µL pipet tips

▪ Reagents Needed per 1 L

(Solution Recipes described below procedure)

- 330 mL Mineral Solution A
- 330 mL Mineral Solution B
- 25 mL Water Soluble Vitamin Solution
- 5 mL Folate-Biotin Solution
- 5 mL Riboflavin Solution
- 50 mL Soluble Carbohydrate Solution
- 100 mL Pectin Solution
- 10 mL Short Chain Fatty Acid Solution
- 0.16 mL Mercaptoethanol
- 0.5 g Cysteine HCl Monohydrate
- 10 mL Urea Solution
- 134.34 mL Autoclaved Distilled H₂O

▪ Reagents Needed per 6 L

- 1980 mL Mineral Solution A
- 1980 mL Mineral Solution B
- 150 mL Water Soluble Vitamin Solution
- 30 mL Folate-Biotin Solution
- 30 mL Riboflavin Solution
- 300 mL Soluble Carbohydrate Solution
- 600 mL Pectin Solution
- 60 mL Short Chain Fatty Acid Solution
- 0.96 mL Mercaptoethanol
- 3.0 g Cysteine HCl Monohydrate
- 60 mL Urea Solution
- 806.04 mL Autoclaved Distilled H₂O

- Measure out all reagents and add to a sterile flask
 - Keep flask covered with tin foil in between adding reagents
- Cover flask with tin foil and mix solution on a stir plate for ~15 min, using a sterile stir bar.
- Warm the media to 37°C prior to mixing with feces.

Solution Recipes

Make these solutions < 1 week before use:

- Mineral Solution A
 - *Make one 2 L batch and one 1 L batch for preparation of other reagents.*
 - Reagents to make **1 L** of solution
 - 5.4 g Sodium Chloride (NaCl)
 - 5.4 g Ammonium Sulfate [(NH₄)₂SO₄]
 - 2.70 g Potassium Phosphate Monobasic Anhydrous (KH₂PO₄)
 - 0.12 g Magnesium Chloride, hexahydrate (MgCl₂*6H₂O)
 - 0.18 g Calcium Chloride dihydrate (CaCl₂*2H₂O)
 - 0.06 g Manganese Chloride tetrahydrate (MnCl₂*4H₂O)
 - 0.06 g Cobalt Chloride hexahydrate (CoCl*6H₂O)
 - Distilled Water
 - Reagents to make **2 L** of solution
 - 10.8 g NaCl
 - 10.8 g (NH₄)₂SO₄
 - 5.40 g KH₂PO₄
 - 0.24 g MgCl₂*6H₂O
 - 0.36 g CaCl₂*2H₂O
 - 0.12 g MnCl₂*4H₂O
 - 0.12 g CoCl*6H₂O
 - Distilled Water

- Mineral Solution B
 - Reagents to make 2 L of solution
 - 5.4 g Potassium Phosphate Dibasic Anhydrous K_2HPO_4
 - Using sterile tools, weigh each reagent and add to a sterile 1 L volumetric flask
 - Add ~500 mL distilled water to the flask and swirl mix until all reagents are completely dissolved
 - Add distilled water until the volume reads 1 L
 - Place a sterile stir bar in the flask and mix on the stir plate
 - Transfer solution to a sterile 1 L Pyrex jar for storage in the refrigerator

- Urea Solution
 - Reagents to make 65 mL
 - 10.83 g urea
 - Distilled Water
 - Weigh the urea and add to a sterile 200 mL bottle.
 - Add 65 mL DI water to the bottle and swirl to mix until all of the urea is completely dissolved.

Make these reagents < 2 days before use:

- Vitamin B-12 Solution
 - Reagents to make 100 mL of solution
 - 0.0025 g Vitamin B-12 (Rovimix B12, 10 mg B-12/g)
 - Distilled Water
 - Weigh the vitamin B-12 and add to a sterile 100 mL amber colored volumetric flask
 - You may need to rinse the weight paper with distilled water to get all of the vitamin B-12 into the flask
 - Add ~50 mL distilled water to the flask and swirl to mix
 - Add distilled water until the volume reads 100 mL
 - Transfer the solution into a dark colored glass bottle. This solution is light sensitive. Store in refrigerator.

- Water Soluble Vitamin Solution
 - Reagents to make 1 L of solution
 - 0.1 g Thiamin HCl (Rovimix B1, 920 mg thiamin/g)
 - 0.01 g Pantothenic Acid (Rovimix Calpan, 920 mg pantothenic acid/ g)
 - 0.1 g Niacin (Rovimix Niacin, 995 mg niacin/g)
 - 0.1 g Pyridoxine (Rovimix B6, 820 mg pyridoxine/g)

- 0.005 g P-Aminobenzoic Acid
 - 10 mL Vitamin B-12 Solution
 - Distilled Water
 - Weigh each reagent and add to a sterile 1 L volumetric flask
 - Add ~500 mL distilled water to the flask and swirl to mix until all reagents are completely dissolved
 - Add the vitamin B-12 solution and mix
 - Add distilled water until the volume reads 1 L
 - Transfer solution to sterile 1 L Pyrex jar. The solution is light sensitive so the jar must be covered with tin foil.
 - Store in refrigerator.
- Folate-Biotin Solution
 - Reagents to make 1 L of solution
 - 0.01 g Folic acid (Rovimix Folic 80 SD, 800 mg/g)
 - 0.002 g Biotin (Rovimix Biotin, 10 mg/g)
 - 0.1 g Ammonium Carbonate
 - Distilled Water
 - Weigh each reagent and add to a sterile 1 L volumetric flask
 - Add ~500 mL distilled water to the flask and swirl to mix until all of the reagents are completely dissolved then add distilled water until the volume reads 1 L
 - Transfer to sterile 1 L Pyrex Jar and cover with tinfoil.
 - Store in refrigerator.
- Riboflavin Solution
 - Reagents to make 100 mL of solution
 - 0.001 g Riboflavin
 - 0.13 HEPES
 - Distilled Water
 - Weigh each reagent and add to a sterile 100 mL amber volumetric flask
 - Add ~50 mL distilled water and swirl to mix until all reagents are completely dissolved
 - Add distilled water until volume reads 100 mL
 - Transfer the solution to a sterile dark colored glass bottle and store in the refrigerator.

Make these solutions the day of use:

- Soluble Carbohydrate Solution
 - Reagents to make 400 mL
 - 5.400 g Maltose
 - 2.696 g Glucose
 - 2.696 g Sucrose

- 20.00 g Soluble potato starch
 - Weigh each reagent and add to a sterile 600 mL beaker
 - Measure 400 mL of Mineral Solution A in a graduated cylinder and pour into beaker
 - Cover beaker with tin foil and mix solution using a sterile stir bar on low heat setting.
 - Must continually stir until and during use – not everything will go into solution.
- Pectin Solution
 - Pre-Heat 600 mL of Mineral Solution A to 70°C
 - Reagents to make 600 mL (*Need 600 ml*)
 - 15.90 g Citrus Pectin
 - Weigh pectin
 - Measure 600 mL of heated (70°C) Mineral Solution A and pour into a sterile 1 L beaker
 - Stir solution vigorously and **slowly** add in the pectin, otherwise it will clump.
 - Allow solution to mix, on medium-low heat, for 1 h

Make this solution immediately before use:

- Short Chain Fatty Acid Solution
 - Use the fume hood
 - Reagents to make 100 ml
 - 17 mL glacial acetic acid
 - 6 mL propionic acid
 - 4 mL n-butyric acid
 - 1 mL isobutyric acid
 - 1 mL n-valeric acid
 - 1 mL iso-valeric acid
 - 1 mL DL- α -methylbutyrate
 - 6 N Sodium Hydroxide
 - Pipette each short chain fatty acid into a sterile 100 mL volumetric flask
 - Add ~50 mL of DI water and swirl to mix, then add DI water until the volume = 100 mL
 - Transfer solution to a sterile 200 mL beaker, add sterile stir bar and stir while adjusting pH
 - Adjust solution to pH 7.0 with sodium hydroxide, add drop by drop very slowly to allow pH to fully adjust between each drop. (takes ~40ml to increase pH to 7)

Starting the Continuous Culture:

Days ahead of time:

1. Autoclave all tubing used in the experiment.
2. Calibrate the pumps for a 3.8% dilution rate.

Fermenter Volume (mL)	1460	5% Margin of Error	
		- 5%	+ 5%
Dilution Rate (%/h)	3.8	52.71	58.25
Dilution Volume (mL/h)	55.48	52.71	58.25
Dilution Volume 1 min.	0.92	0.88	0.97
Dilution Volume 3 min.	2.77	2.64	2.91
Dilution Volume 5 min.	4.62	4.39	4.85
Dilution Volume 10 min.	9.25	8.78	9.71
Dilution Volume 20 min.	18.49	17.57	19.42
Dilution Volume 24 hr.	1331.52	1264.94	1398.10

3. Make buffer

RO H₂O - 87.3 L
Prebuffer A - 12 L
Prebuffer B - 0.60 L
L-Cys Hcl - 25 g

Pr

Bubble CO₂ through until pH decreases to 7.0 ± 0.05.

Prebuffer A				
	Conc. (g/L) PreBuffer A	Conc. (g/L) in Final Soln	g/batch	g/Total Vol Req
Na ₂ CO ₃	31.00	3.72	372	1488
K ₂ CO ₃	40.17	4.82	482	1928
KH ₂ PO ₄	11.25	1.35	135	540
Na ₂ HPO ₄	18.33	2.20	220	880

Prebuffer B			
	Conc. (g/L) PreBuffer B	Conc. (g/L) in Final Soln	g/Total Vol Req
MgCl ₂ *6H ₂ O	12.00	0.07	30.0
NaCl	47.00	0.28	117.5
KCl	57.00	0.34	142.5
CaCl ₂	5.33	0.03	13.3
Cys-HCL	g per batch		
	25		

During the 6 h pre-incubation period, begin setting up the equipment:

Equipment and Needed:

- 2 large stir plates
- 2 large sterile stir rods
- 2 large containers to mix and dispense fermenter inoculant
- 2 disinfected plastic graduated cylinders.

1. Turn on water bath heaters to 39°C.
2. Connect all tubing.
3. Fill buffer tanks and make more buffer
 - a. Pre-warm 29 L of buffer to 39°C
4. Cut cheesecloth to strain fecal inoculum.
5. Clean and disinfect 2 separate containers to mix and dispense fermenter inoculant and 2 stir rods.

Keep all samples, materials, equipment, and gloves completely separate between horse and rhinoceros samples

6. After pre-incubation is done, bring the flasks with the fecal inoculum to the continuous culture room.
7. Strain the feces solution through 4 layers of cheesecloth.
8. In two separate and disinfected containers, mix 3600 ml of each feces solution with 14,400 ml of pre-warmed buffer. (Achieves a 1:4 ratio of feces inoculum: buffer)
9. Mix for 5 minutes before measuring out 1460 mL to place in each fermenter. Make sure different graduated cylinders are used for horse vs. rhino.
10. After filling each fermenter, place the lid and connect all tubing.
11. Turn on CO₂ at equivalent pressure within each fermenter.
12. Turn on peristaltic pumps.

- Fermenters are fed at exactly 8 am and 8 pm.
- Routine Maintenance Checks:
 - Buffer tanks filled
 - All fermenters mixing
 - Water bath is at 39°C
 - Peristaltic pumps are set at calibrated mark
 - CO₂ is flowing equally to all fermenters and tanks are not empty
 - All stoppers are in place
 - Effluent hoses are not clogged and are in appropriate collection vessel

***In Vitro* Foregut Digestion Procedure**

Based on methods by Boisen and Fernhdez (1997) Murray et al. (2003) and Sweeney, (2012)

Continuous culture diets were subjected a simulated foregut digestion procedure to mimic the feed form presented to cecal microbes *in vivo*.

1. Weigh out 17.5 g of feed ahead of time.

Digestion Reagents:

- Artificial Saliva Solution
 - Prepare < 48 h ahead of time
 - Add the following amounts to 2 L dH₂O and bring to pH 7 with CO₂
 - Na₂HPO₄ - 0.1420 g
 - MgCl₂ • 6 H₂O - 0.3049 g
 - CaCl₂ • 2 H₂O - 1.4700 g
 - KCl - 1.1184 g
 - NaHCO₃ - 4.0325 g
 - NaCl - 1.5000 g
 - Urea - 0.0901 g
 - Mucin - 0.5000 g
 - α – amylase (1.5 U/mg) - 0.3333 g
 - Gliadin - 0.5000 g
 - Collagen - 0.5000 g
- Phosphate Buffer
 - Weigh 2.1 g of Sodium Phosphate Dibasic, Anhydrous and add to a 1 L volumetric flask
 - Add 11.76 g Sodium Phosphate Monobasic, Monohydrate to the flask
 - Add DI water until the volume reads 1 L
 - Add a stir bar to the flask and place on the stir plate. Mix well.
 - Check the pH. Target = 6.8 ± 0.1.
 - Transfer to a Pyrex jar and store in the refrigerator until use.
- Pepsin Solution
 - * Prepare the day of use
 - Formulated to contain 50 mg pepsin / ml
 - Dissolve 2.5 g pepsin in 50 mL of 0.1 mol/L HCl
 - Optimum pH = 2 – 4

- Pancreatin Solution (100 mg pancreatin / ml)
 - *Yields ~60% of the volume of buffer added
 - *Prepare the day of use:
 - Weigh 100 g of porcine pancreatin and add 100 ml of phosphate solution.
 - Place on stir plate and mix gently until the pancreatin is dissolved
 - Transfer to 50 ml centrifuge tubes with lids – seal and store until ready to use.
 - Centrifuge in the 50 ml tubes at 1800 x g for 10 min just before use
 - Pour supernatant into clean 600 ml beaker and store until ready to use, discard particulate material.

1. Preparation

- a. Prepare all solutions just before beginning incubation.
- b. Turn on a shaking water bath to 39°C

2. Salivary Incubation

- a. Add 17.5 g of feed to a 500 ml Pyrex jar.
- b. Add 35 ml of artificial saliva and a clean stir bar.
- c. Place in 39°C water bath in continuous culture room and continually stir throughout procedure.
- d. Add 140 ml of the phosphate buffer solution.

3. Peptic Digestion

- a. Monitor pH, add 0.5 M HCl until pH drops to 2 ± 0.1 (~60 ml)
- b. Add 17.5 ml of the pepsin solution.
- c. Cap tightly and allow to mix in water bath for 2 h.

4. Intestinal digestion

- a. After 2 h add 0.5 M NaOH until the pH rises to 6.8 ± 0.1 (~105 ml)
- b. Add 8.75 ml of the pancreatin solution and mix for 2 min.
 - i. Provides 100 mg pancreatin / g of feed
- c. Cap the jar allow to digest in water bath for 4 h.

5. Precipitation

- a. After 4 h add 140 ml of 95% ethanol, continue to mix for 5 min.
- b. Remove jars from the water bath and let sit at room temperature for 1 h
- c. Swirl to mix then pour into 250 ml centrifuge containers and balance the containers with the lids before centrifuging for 10 min at 6900 x g and 4°C.
- d. Label and record the weight of a large Dacron bag
- e. Remove supernatant by vacuum aspiration and filter through the Dacron bag to collect feed particles.
- f. Wash the pellets and strain through the same Dacron bag to collect all undigested feed particles.
- g. Dry the bags in a 55°C oven for 24 h.
- h. Weigh the bags and dried digested feed.
- i. Collect feed into a pre-weighed 8oz container.

Continuous Culture Sampling and Termination Procedure

After a successful 5 d adaptation of the continuous culture fermenters to the experimental diets, the fermenters and effluent will be sampled for 3 d.

Diet Samples

- Triplicates of each diet will be analyzed separately for each continuous culture.
- Dry Matter
- Organic Matter
- Crude Protein
- Neutral Detergent Fiber and Acid Detergent Fiber
- Iron
- Protein Precipitable Phenolics

Fermenter Sampling

- Samples will be taken at 0 h (immediately prior to), 2, 4, 8 and 12 h after feeding.
 - At these times, pH will be recorded as well. (Make sure to sample all of 1 inoculum source at 1 time, rinse probe with sterile DI water between fermenters and sterilize probe in between inoculum sources)

Materials and Equipment Needed:

- Pipet aid and 10 ml pipet tips
- 120, 50 ml centrifuge tubes
- 6 N HCl
- Ice bath

- Before sampling, make sure all fermenters are being mixed well. Using a new pipet tip for each fermenter, draw up 10 ml from the middle of the fermenter liquid volume.
- Dispense samples into a labeled 50 ml centrifuge tube.
- In the tube (for NH₃ and VFA analysis) add the appropriate volume of 6 N to decrease the pH of the sample to at least pH 3
 - Mix 5x by inversion.
 - Immediately place the tube in an ice bath
- Store all tubes into a -20°C freezer after sampling.
- Fermenter samples will be composited each sampling day, by hour.

Sample Preparation and Analysis

Ammonia and VFA analysis

- Thaw the tube containing acidified fermenter contents in a 39°C water bath immediately prior to use.
- Mix contents using a vortex mixer.
- Centrifuge the tubes at 10,000 x g for 10 min.

- Subsample 2 ml of the supernatant into a microcentrifuge tube for NH₃ analysis using the Phenol-Hypochlorite Procedure of Broderick and Kang (1980)
- Subsample 5 ml of the supernatant into a 15 ml centrifuge tube and follow the VFA procedure by Galyean and May (2010, pg. 160-162)
 - o Including addition of metaphosphoric acid and 2-ethyl butyric acid as an internal standard followed by further centrifugation.

Effluent Sampling:

Materials/Equipment needed

- 24, 5-gallon collection vessels
- Funnel
- Pipet aid
- 24, 25 ml pipet tips
- 24, 50 ml centrifuge tubes
- Ice bath

- Before feeding in the morning, remove the effluent collection cylinders from the ice and record effluent level (make sure to remove tubing when recording measurement).
- Stopper the end of the cylinder with a plastic cup and swirl to mix the contents well and take a 15 ml subsample using the pipet.
- Dispense the sample into the 50 ml centrifuge tubes and immediately place on ice.
- After sampling store the tubes in a -20°C freezer.
- Using the funnel, pour the remaining effluent contents into the respective collection vessel, making sure to swirl at the end to collect all of the feed material.
- Samples will be composited over each day.

Sample Preparation and Analysis

- Label and weigh clean dry, containers to freeze-dry the samples
- Shake effluent contents well then pour 600 ml into freeze-drying container

- Weigh the container + effluent contents without the lid
- Place cheese cloth over the top, secure with a rubber band, and place in the -20°C freezer until ready to lyophilize.

Analyze dry samples for:

- | | |
|------------------|----------------------------------|
| - Dry matter | - NDF & ADF |
| - Organic Matter | - Purines |
| - Nitrogen | - Protein Precipitable Phenolics |

Experiment termination procedure:

- On the last day of sampling after the final effluent collection, take 2 samples for quantification of microbes, and microbial population analysis and functional characterization.

Materials and Equipment Needed:

- Pipet aid
- 24, 50 ml pipet tips
- 96, 50 ml centrifuge tubes (48 must be sterile for microbial analysis)
- Ice bath
 - Before sampling, make sure all fermenters are being mixed well.
 - Maintaining as sterile technique as possible, using a pipet with a new pipet tip for each fermenter, draw up 40 ml from the middle of the fermenter liquid volume.
 - Dispense each of the samples into separate, sterile, labeled 50 ml centrifuge tube.
 - Immediately place each tube into the ice bath.
 - Store the tubes for microbial population analysis in a -80°C freezer after sampling.

- Whole fermenter content collection:

Materials and Equipment Needed:

- Industrial Blender
- Cheese cloth (4 layers/ fermenter)
- 24 half-gallon collection vessels, labeled with lids
- Funnel

Procedure

- Shut off stir plates and remove stir bars from each fermenter.

- Pour fermenter contents into blender and mix for 30 seconds.
- Pour through 4 layers of cheesecloth and collect in the half-gallon containers.
- As soon as all 24 are collected, store in the -20°C.

APPENDIX C – EFFECT OF GRAPE SEED EXTRACT ON IRON ABSORPTION BY INTESTINAL EPITHELIAL CELL LINE 6

Introduction

Absorption of dietary iron is a complex process mediated by specialized transporters and exporters in the cell and is subject to hormonal control. At the apical surface of absorptive enterocytes in the duodenum, ferric iron (Fe^{3+}) is reduced by duodenal cytochrome B to ferrous iron (Fe^{2+}) that is then transported across the membrane by divalent metal transporter-1 (**DMT1**). Intracellular Fe^{2+} is then stored as ferritin in the cytoplasm or exported to the basolateral surface to plasma, via ferroportin. In this membrane, ferroxidase hephaestin catalyzes the oxidation back to the ferric form. Ferric iron is then taken up by plasma transferrin for distribution to tissues (Papanikolaou and Pantopoulos, 2005; Valerio, 2007; Ganz and Nemeth, 2012).

Measuring iron bioavailability in animal models is a lengthy, complex, and expensive process. For this reason *in vitro* methods have been developed as a rapid screening model to predict potential iron available for absorption. Earlier *in vitro* studies utilized simulated gastrointestinal digestion of a meal and measured iron dialyzability which does not give a complete picture of bioavailability (Kane and Miller, 1984; Forbes et al., 1989; Luten et al., 1996). To address this problem cell culture methods have been developed that more accurately measure iron absorption.

Intestinal epithelial cell line 6 (**IEC-6**) is derived from rat small intestine and develops enterocyte like properties upon reaching confluence. IEC-6 has been shown to express genes for DMT1, iron-regulated transporter1/ferroportin, and ferritin as well as

functional uptake and efflux transporters (Nichols et al., 1992; Simovich et al., 2002; Thomas and Oates, 2004; Thomas and Oates, 2004). Thomas and Oates (2002) validated the use of the IEC-6 cells as a model of intestinal iron transport by demonstrating the expression of functional iron transport proteins and that they are regulated by variation in cellular iron. IEC-6 has also been used in studies evaluating polyphenolics properties (Yang et al., 2001; Iyengar et al., 2010; Oz and Ebersole, 2010). This cell line was used in this research due to its validity as a model for iron absorption, transport and storage. Moreover, IEC-6 most closely resembles the cells at the site of iron absorption in the rhinoceros small intestine.

A different cell line, Caco-2, is frequently used as a model of human iron physiology. Caco-2 cells were derived from a human colorectal adenocarcinoma, and develop enterocyte like properties after reaching confluence. This model is widely used for pharmaceutical, biochemical, and toxicological studies as well as for investigation of iron transport (Glahn et al., 1998; Glahn and Wortley, 2002) and has been used simultaneously with IEC-6 cells with comparable results (Thomas and Oates, 2004). As in the animal intestine, both IEC-6 and Caco-2 cells display enhanced transepithelial transport of iron in iron-depleted cells and express DMT1, duodenal reductases, ferroportin, hephaestin, transferrin receptor and form ferritin (Han et al., 1995; Han et al., 1999; Martini et al., 2002). Glahn et al. (1998) utilized the Caco-2 cell line in conjunction with *in vitro* digestion techniques and have developed a model in which foods undergo simulated gastric digestion followed by intestinal digestion in the presence of the cell monolayer. This model measures iron solubility in addition to providing a measure of uptake and is a great advancement over the use of *in vitro* digestion alone.

The technique developed by Glahn is unique in that formation of ferritin by cells exposed to digesta serves as an indicator of bioavailable iron, instead of measuring radiolabeled iron. An increase in cell ferritin is undisputable evidence that iron has entered the cell, whereas an increase in the apparent cellular content of a radio iron tracer could represent surface-bound iron as well as intracellular iron. This model has been extensively utilized to study iron absorption and of particular interest to this research, inhibition of iron absorption by polyphenolics (Glahn and Wortley, 2002; Yun et al., 2004; Laurent et al., 2007; Kim et al., 2008; Ma et al., 2010). An ELISA assay was used to quantify the amount of stored ferritin following iron and/or GSE treatment.

Although many studies have used cell culture models to show decreased iron absorption due to polyphenolics, it is important to complete this type of test with those present in grape seed extract, as these compounds are widely varied in structure and iron chelation ability. We hypothesized that increasing levels of GSE would decrease iron absorption consequently decreasing total iron and ferritin concentration.

Cell Culture General Information and Protocols

Description of ingredients in media

- Eagle's Minimum Essential Medium (MEM)
 - Simple medium with basic required nutrients
 - Contains reduced sodium bicarbonate concentration for use with 5% CO₂
- Dulbecco's Modified Eagle's Medium (DMEM)
 - Standard culture media that has about 2x the concentration of amino acids and 4x the vitamins as MEM, as well as ferric nitrate, sodium pyruvate, and supplementary amino acids.
 - Contains 4,500 mg/L glucose and reduced sodium bicarbonate concentration for use with 5% CO₂
- Fetal Bovine Serum
 - Serves as a source for amino acids, proteins, vitamins, carbohydrates, lipids, hormones, growth factors, minerals and trace elements.
 - Also buffers the culture medium, inactivates proteolytic enzymes and conditions the growth surface of the culture vessel.
 - Storage:
 - -20°C or colder for storage over 30 days. Avoid repeated freeze-thaws by dispensing and storing in aliquots.
- HEPES
 - Buffers the pH of the medium.
 - This compound can be toxic to cells so evaluate its effects before use.
 - Has been shown to greatly increase the sensitivity of media to the phototoxic effects induced by exposure to fluorescent light.
- Phenol Red
 - Used to monitor the pH of media.
 - During cell growth, the medium changes color as it changes pH due to metabolites released by the cells.
 - Low pH = yellow
 - High pH = purple
 - Ideal pH (7.4) = bright red
- L-Glutamine
 - Essential amino acid that is very labile so it is often omitted from commercial liquid medium preparations to lengthen shelf life.
 - If L-glutamine is suspected to be a limiting factor during cell growth, add a small amount (~2 mM final concentration) to the culture medium. If the cell growth rate increases, L-glutamine is likely to be deficient and more should be added.
 - Concentration in DMEM is 4 mM.

- Don't add more than called for in the original medium formulation.
 - L-glutamine degradation results in the build-up of ammonia, toxic to cells.
- Antibiotics (Gentamicin)
 - Added to cell culture media as a prophylactic to prevent contamination or as a cure once contamination is found.
 - Routine use is not always recommended because they can mask contamination by mycoplasma and resistant bacteria.
 - Gentamicin sulfate is used at 50-100 µg/mL

Preparation of Growth and Maintenance Medium

1. Dulbecco's Modified Eagle's Medium (DMEM) – Using Invitrogen RPMI Custom version
 - a. Add FBS to achieve a concentration of 10% (v/v) of the whole solution
 - i. 55 ml / 500 ml Media
 - b. Add antibiotic solution to achieve a concentration of 1% (v/v) of whole solution
 - i. 4.58 ml of 100x Penicillin/Streptomycin solution = 50 mg/L penicillin, 83 mg/L streptomycin
 - c. Add 0.1 unit / mL of bovine insulin (90%)
 - i. 1.85 mg / 500 ml media = 0.1 U / ml
 - d. Add 0.11 ml fungizone = 0.5 µg/ml
2. Eagle's Minimum Essential Medium (MEM)
 - a. 10 mmol/L PIPES (1.5119 g)
 - b. 1% antibiotic solution (4.58 ml 100x penicillin/streptomycin)
 - c. hydrocortisone (2mg/ 500 mL)
 - d. insulin (2.5 mg/500 mL)
 - e. selenium (2.5 µg/500 mL)
 - f. triiodothyronine (17 µg/500 mL)
 - g. epidermal growth factor (10 µg/500 mL = 100 µl of reconstituted 0.1 mg/ml EGF solution)
 - h. fungizone (0.15 ml/500 ml)

Culture Vessels and Surfaces

- 75 cm² flasks use a working media volume of 15-25 mL and can yield 7.5×10^6 cells (based upon a density of 1×10^5 cells/cm²).
- 6-well plates must be used with incubators that control humidity and CO₂ levels.
 - o Growth well: 9.40 cm²
 - o Working volume / well: 2.0 to 3.0 mL
 - o Cell yield: 9.5×10^5 cells

Aseptic Technique

1. Sanitize the hood using 70% ethanol before starting work
2. Sanitize gloves by washing them in 70% ethanol and allowing to air dry for 30 seconds
3. Equipment in the hood or that which will be taken into the hood during the procedures (media bottles, pipette tip box, pipettes) should be wiped with a tissue soaked in 70% ethanol prior to use.
4. Make sure all materials and equipment needed are in the hood before starting work, and make sure all have first been sanitized using 70% ethanol
5. While working, do not contaminate hands or gloves by touching anything outside the hood (especially face and hair). If glove becomes contaminated re-sanitize with 70% ethanol as before.
6. Movement within and immediately outside the cabinet must not be rapid. Slow movement will allow the air within the cabinet to circulate properly
7. Speech, sneezing and coughing must be directed away from the cabinet so the airflow is not disrupted.
8. After you're done, disinfect all equipment and material before removing from the hood. Spray the work surfaces inside with 70% ethanol and wipe dry with tissue. Dispose of the tissue by autoclaving.
9. Discard gloves after handling contaminated cultures and at the end of all procedures.
10. Sanitize the hood with 10 – 30 min UV light. Ensure they are not left on for extended periods.

Subculturing

Equipment:

- Incubator at 37°C with a 5% CO₂/95% air atmosphere, 90% humidity
- Inverted Phase contrast microscope
- Laminar flow hood

Materials:

- Nonsterile
 - Pipetting aids
 - Disposal tray or bucket for pipettes
 - Bottle of 70% ethanol for wiping down work area
 - Paper towels
 - Marking Pen
 - Liquid waste container
- Sterile
 - Flask of actively growing cells that are 80 to 90% confluent
 - Complete growth medium, 37°C
 - PBS – Ca²⁺ and Mg²⁺ free, 37°C
 - TrypLE express solution, 37°C
 - Appropriate culture vessels
 - 1, 5, 10 and 25 mL pipets and pipette tips

The amounts in this procedure are for a 75-cm² flask

1. Bring the TrypLE solution, PBS without Ca or Mg and growth medium to 37°C.
2. Add 12 mL to 15 mL of fresh culture medium to a new flask and equilibrate this medium to the appropriate pH and 37°C.
3. Aspirate and discard the cell culture medium from the flask
4. Rinse the cell monolayer with 5 – 10 mL of PBS without Ca or Mg – use approx. half the volume of culture medium.
 - a. Slowly rock it back and forth to remove all traces of serum.
 - b. Repeat if cells are strongly adherent.
 - c. Remove and discard the PBS wash.
5. Add 3 – 5 mL of TrypLE (use ~1 mL / 25 cm² surface area), rotate flask to cover monolayer with trypsin, and incubate at 37°C for about 5 minutes
 - a. Watch for the media to become cloudy.
 - b. Tap the sides of the flask to help dislodge cells.

6. Once the cells appear to be detached (They will appear rounded and refractile under the microscope), using a 10 mL pipet add 6 to 8 mL of serum containing - growth medium with a pipette to the cell suspension.
 - a. Wash any remaining cells from the growth surface of the flask.
 - b. Check the cells with the microscope to be sure that most (>95%) are single cells. If cell clusters are apparent, continue to disperse the cells with gentle pipetting.
7. Divide the suspension according to the appropriate 1:3 or 1:6 split ratio and dispense them into the medium of the newly prepared flask.
 - a. Do not add a concentrated cell suspension to an empty culture vessel as this can result in uneven cell attachment and growth.
8. Place the flask back into the incubator.
9. Examine the culture the following day to ensure the cells have reattached and are actively growing.
10. Change the medium every two days.

Examining the culture

View cultures using an inverted microscope to assess the degree of confluence and confirm the absence of bacterial and fungal contaminants.

- pH:
 - color of phenol red indicator.
 - Yellow = acidic, Purple/Fuchsia = alkaline
 - In general, cells can tolerate slight acidity better than a pH above 7.6
- Cell Attachment:
 - Are most of the cells well attached and spread out?
 - Are the floating cells dividing cells or dying cells which may have an irregular appearance?
- Rate of attachment:
 - Attachment within an hour or two suggests that the cells have not been traumatized and that the in vitro environment is not a grossly abnormal.
 - Longer attachment times are suggestive of problems.
 - However, good cultures may result even if attachment does not occur for four hours.
- Percent Confluent:
 - The growth of a culture can be estimated by following it toward the development of a full cell sheet (confluent culture)
 - By comparing the amount of space covered by cells with the unoccupied spaces you get an estimate of percent confluent.
- Cell Shape
 - Round cells in an uncrowded culture is not good unless there happen to be dividing cells.
 - Look for doublets or dividing cells.
 - Get to know the effect of crowding on cell shape
- Cell Size
 - Look for giant cells
 - The number of giant cells will increase as a culture ages or declines in “well-being”. The frequency of giant cells should be relatively low and constant under uniform culture conditions.
- Microbial Contamination

- Usually occurs within a few days and is typically obvious to the naked eye.
- Changes in medium: turbidity, presence of particles visible in suspension, rapid decline in pH (yellow color)

Cell Counts and Viability

Equipment:

- Hemacytometer
- 37°C incubator

Materials:

- 70% ethanol
- Cell cultures
- Sterile 1x PBS
- Trypsin/EDTA, 37°C
- Complete Medium with serum
- Sterile Pasteur pipets
- Hemacytometer cover slip
- Hand-Held Counter
- 0.4% trypan blue (w/v)
- HBSS

Preparation

1. Bring the 0.25% trypsin- 0.53 mM EDTA solution and PBS without Ca or Mg to 37°C.
2. Remove and discard the cell culture medium from the flask
3. Rinse the cell monolayer with PBS without Ca or Mg – use approx. half the volume of culture medium. Repeat if cells are strongly adherent. All serum must be removed before the addition of trypsin. Remove PBS wash.
4. Add 2 mL to 3 mL of the trypsin-EDTA solution (use ~1 mL / 25 cm² surface area), rotate flask to cover monolayer with trypsin, and incubate at 37°C for about 10 minutes
 - a. Watch for the media to become cloudy.
 - b. Check the progress of cell dissociation by microscopy. To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for them to detach.
 - c. Cells should only be exposed to Trypsin/EDTA long enough to detach cells. Prolonged exposure could damage surface receptors.
5. Once the cells appear to be detached (They will appear rounded and refractile under the microscope) add 6 to 8 mL of serum containing - growth medium with a pipette to the cell suspension to inactivate the trypsin.
 - a. Gently wash any remaining cells from the growth surface of the flask.

- b. Check the cells with the microscope to be sure that most (>95%) are single cells.
6. Dilute cells as needed to obtain a uniform suspension. Disperse any clumps with gentle pipetting.

Load hemacytometer

7. Use a sterile Pasteur pipet to transfer cell suspension to edge of hemacytometer counting chamber. Hold tip of pipet under the coverslip and dispense one drop of suspension.
 - a. The suspension will be drawn under the coverslip by capillary action.
8. Fill the second counting chamber.

Count Cells

9. All cells to settle for a few minutes before beginning count. Blot off excess liquid.
10. View slide on microscope with 100 x magnification. Position slide to view the large central area of the grid. This area is bordered by a set of three parallel lines. The central area of the grid should almost fill the microscope field.
11. Use a hand-held counter to count cells in each of the four corners and central squares. Repeat for the other counting chamber. Five squares are counted from each of the two counting chambers for a total of 10 squares counted. Count cells touching the middle line of the triple line on the top and left of the squares. Do not count cells touching the middle line of the triple lines on the bottom or right of the square.

Calculate Cell Number

12. Determine cells per mL by the following calculations:
 - a. $\text{Cells/mL} = (\text{avg. count per square}) \times \text{dilution factor} \times 10^4$
 - b. $\text{Total cells} = (\text{cells/mL}) \times \text{total original volume of cell suspension from which sample was taken.}$

Viability

1. Determine number of viable cells by adding 0.5 mL of 0.4% trypan blue, 0.3 mL HBSS, and 0.1 mL cell suspension to a small tube.
2. Mix thoroughly and let stand for 5 min. before loading hemacytometer.

3. Nonviable cells will take up the dye while live cells will be impermeable to the dye.
4. Count total number of cells and total number of viable (unstained) cells.
5. Calculate percent viable cells:
 - a. $\% \text{ viable} = (\# \text{ unstained cells} / \text{total } \# \text{ cells}) \times 100$
6. Clean coverslip and hemacytometer by rinsing with 70% ethanol followed by deionized water.

Assessment of IEC-6 monolayer permeability using phenol red flux

Based on methods by (Jovov et al., 1991) with modifications by (Forsythe et al., 2002)

Reagents:

- DMEM media without phenol red warmed to 37°C
- Phenol red containing cell culture media warmed to 37°C

Equipment:

- incubator at 37 °C
- laminar flow hood
- spectrophotometer
- pipets and tips
- sterile 2 ml microcentrifuge tubes

Procedure:

- 1) Remove all media from both apical and basolateral chambers
- 2) Rinse the monolayer with 1ml pre-warmed DMEM without phenol red
- 3) Pipet 1 ml DMEM without phenol red into the basolateral compartment
- 4) Pipet 2ml of pre-warmed phenol red containing media into the insert
- 5) Incubate at 37 °C for exactly 3h (record timing for each plate)
- 6) Collect all liquid from the basolateral compartment into a sterile, labeled 2ml microtube
- 7) Read absorbance on a spectrophotometer at a wavelength of 479 nm using the fixed program
 - a. Use an aliquot of DMEM without phenol red as a blank
 - b. Read an aliquot of the phenol red containing media as an upper control
- 8) Calculate phenol red flux using the following equation:

$$J_{PR} = \frac{(A_{479} \times vol_a)}{(t \times A \times EC)}$$

- J_{PR} = Phenol red flux
 - A_{479} = absorbance at 479 nm
 - Vol_a = apical compartment initial volume
 - t = time
 - A = surface area (24 mm² for a 6-well plate)
 - EC = extinction coefficient (8450 L/mol/cm for phenol red)
- 9) Once low and consistent phenol red flux values are obtained, confluence has been reached, tight junction are formed and you can begin the experiment.

Experiment Procedures

Based on methodology by Glahn et al. (1998) and Glahn and Wortley (2002)

Experiment 1. Tannin and Iron Solutions – No digestion

Replicate experiment three times.

Materials

- Incubator at 37°C with a 5% CO₂/95% air atmosphere, 90% humidity
- Rocking platform tabletop shaker
- Temperature monitor/recorder
- 6-well plates with Transwell inserts, 0.4µm pore size, polyester, tissue culture treated, 4.67 cm² growth area (Corning, Inc.)
- Pipets and tips
- Serological pipets and aid
- Sterile, acid washed Beakers
- Sterile, acid washed stir bars
- Vortex
- 70% ethanol
- Sterile DI Water
- PBS
- TrypLE Express (Invitrogen)
- Cell scraper
- Bench-top Sonicator
- Supplemented DMEM medium
- Supplemented MEM medium
- Cell harvest rinse solution
- Fe Solution
- Tannin Solutions

Procedure

Seeding and Preparation

1. Add 2.5 mL complete DMEM medium to each plate well then place Transwell insert and add 1.5 mL medium inside each insert.
2. Incubate plate at 37°C for at least 1 hour or even overnight.
3. Remove media from well inserts and add in fresh media (1.5mL) containing ~50,000 cells/cm² to the Transwell inserts.
 - a. Insert membrane growth area = 4.67 cm²
 - b. 233,500 cells total ~ 155,666 cells / mL
4. After seeding, return plates to the incubator and examine the cultures periodically.
5. Change media in both compartments every 2 days
6. Allow cells to grow for ~20 days post confluence. (**Record Passage Number**)
7. Measure and record confluence and tight junction formation using the Phenol Red Flux assay.
 - a. Transwell inserts without IEC-6 cells are treated as a blank.

- b. Once cells have achieved appropriate Phenol Red Flux (J_{PR}) values and tight junctions have formed, begin experiment.

8. Record:

Began experiment _____ days post seeding, phenol red flux values ranged _____.

- Minimum Essential Medium (**MEM**) supplemented with:
 - 10 mmol/L PIPES
 - 1.5119 g PIPES / 500 ml MEM
 - 1% antibiotic
 - 4.58 ml 100x penicillin/streptomycin
 - = 50 mg/L penicillin, 83 mg/L streptomycin
 - hydrocortisone (4mg/L)
 - insulin (5 mg/L)
 - selenium (5 μ g/L)
 - triiodothyronine (34 μ g/L)
 - epidermal growth factor (20 μ g/L)
 - reconstituted at 0.1 mg/ml so 100 μ L / 500 ml MEM were supplemented
 - Fungizone
 - 0.11 ml fungizone / 500 ml MEM = 0.5 μ g/ml

Day of experiment:

Sample and Digestion Solutions Preparation:

- Complete shortly before use
- All glassware used for sample preparation must be acid washed

- Fe Solution
(Provides the same Fe concentration as in the 1 g diet sample digested in exp. 2, approx. 290 μ M)
 - Bring 1L of sterile distilled water to pH 5.5 using 1 N HCl
 - Add 0.0806 g $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ to exactly 500 mL of the acidified distilled water
 - Mix well on a stir plate then cover with foil to prevent light oxidation.
 - Take a sample to analyze for iron concentration.

- Treatment Solutions
(Provides the same tannin concentrations as in the 1 g diet sample digested in cell culture experiment 2)
 - Sterilize the necessary amount of distilled water.

- Add the appropriate amount of GSE or TA to 50 mL of sterilized water and mix well on a stir plate.
- Take a sample of each to analyze for iron concentration.

Treatment	g GSE or TA / 50 mL
Low (1.33%) GSE	0.0443 g GSE
Mid (2.66%) GSE	0.0887 g GSE
High (4.00%) GSE	0.1333 g GSE
Tannic Acid	0.0443 g TA

Experiment Procedure

Treatment Assignments

- Trt 1: Sterile DI H₂O' + Fe Soln'
- Trt 2: Low (1.33%) GSE Soln' + Fe Soln'
- Trt 3: Mid (2.66%) GSE Soln' + Fe Soln'
- Trt 4: High (4.00%) GSE Soln' + Fe Soln'
- Trt 5: Tannic Acid (1.33%) Soln' + Fe Soln'
- Trt 6: High (4.00%) GSE Soln' + H₂O
- Trt 7: MEM only
- Blank inserts (without cells) receive trt 1
 - After measured as a TEER blank, these wells are used to measure soluble iron that dialyzed through the membranes
 - Because a large portion of the iron that passes into the bottom chamber may be taken up by the cells, these wells are used for more accurate measurement of the amount of dialyzable iron

Position	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
A	3	4	5	2	1
B	Blank insert - 1	7	4	7	5
C	6	6	Blank insert - 1	4	2
D	1	Blank insert - 1	3	1	3
E	2	2	1	Blank insert - 1	6
F	4	3	7	5	Blank insert - 1

1. Remove media from both chambers. Decant the media out of the apical chamber to prevent harming the cell layer.
2. Rinse 1x with pre-warmed PBS then 1x with pre-warmed supplemented MEM.

3. Pipet 1 ml MEM into the apical chamber on top of the cell layers. Take a sample of the supplemented MEM for iron analysis. Place plates back into incubator.
4. Mix each treatment solution. Make sure to prepare enough of each treatment for 1.5 ml of sample per well

Treatment	Concentrations per sample
1	1 ml sterile DI H ₂ O + 1 ml Fe soln'
2	1 ml low GSE soln' + 1 ml Fe soln'
3	1 ml mid GSE soln' + 1 ml Fe soln'
4	1 ml high GSE soln' + 1 ml Fe soln'
5	1 ml tannic acid soln' + 1 ml Fe soln'
6	1 ml high GSE soln' + 1 ml sterile DI H ₂ O
7	MEM only

5. Pipette 0.850 ml of each solution to be combined into a 2 ml microcentrifuge tube and vortex to mix.
6. Place the tubes horizontally on the shaker plate in the 37°C incubator for 2 hours. Record the start time for the tubes for each plate.
7. After 2 hours, pipet 1 ml of the appropriate treatment mixture onto the apical chamber of each well. Cover the plate and place back in the incubator for 22 more hours.
8. Termination
 - Collect any media or liquid that dialyzed through the insert membrane of the blank wells.
 - Collect in a microcentrifuge tube and store at -20°C for future iron and polyphenol analysis.
 - Collect any media or liquid from the basolateral compartments in a microcentrifuge tube and store at -20°C for future iron and polyphenol analysis.

Cell Harvest

Materials:

- Deionized water
- Cell scraper
- Bench-top Sonicator
- Tubes to collect cells
- Rinse solution
- PBS

Solution recipes:

- Rinse Solution (140 mM NaCl, 5 mM KCl, 10 mM PIPES)
 - Weigh 3.0237 g PIPES into a sterile, acid washed 2 L flask
 - Add 950 ml sterile DI H₂O and while stirring, drop in 6 N NaOH until pH nears 7 and PIPES goes into solution.
 - Mix using a sterile, acid washed stir bar
 - While mixing, add 8.1816 g NaCl and 0.3727 g KCl to the solution
 - Transfer into a 1 L volumetric flask and adjust volume to 1 L using sterile DI H₂O
 - Check pH, adjust back to 7 if necessary.

Procedure

1. Harvest cells 24 h after initially mixing the treatment solutions.
2. Remove all media or liquid covering the cells by decanting into a waste container.
3. Wash cells with a 2 mL volume of cold PBS solution.
4. Decant PBS.
5. Add 2 mL of rinse solution, swirl to wash then decant liquid off. Repeat 1x.
6. Add 2 mL of DI water and place plate on a rack such that the bottom of each plate is in contact with the water of a bench top sonicator – kept in a cold room at 4°C
7. Sonicate cells for 15 minutes
8. Using a cell scraper, scrape the cells from the insert and harvest along with the 2 mL volume of water in each well and store at -20°C until analysis for cellular protein and ferritin formation.

Experiment 2. Continuous Culture Diet Digestion

Replicate experiment three times.

Materials

- Incubator at 37°C with a 5% CO₂/95% air atmosphere, 90% humidity
- Rocking platform tabletop shaker
- Temperature monitor/recorder
- 6-well plates with Transwell inserts, 0.4µm pore size, polyester, tissue culture treated, 4.67 cm² growth area (Corning, Inc.)
- Millipore Amicon Ultra-15 filtration unit (50ml centrifuge tube size)
- Pipets and tips
- Serological pipets and aid
- Sterile, acid washed Beakers
- Sterile, acid washed stir bars
- Vortex
- 70% ethanol
- Sterile DI Water
- PBS
- TrypLE Express (Invitrogen)
- Cell scraper
- Sonicator
- Porcine pepsin (800-2500 units/mg protein)
- 50 ml centrifuge tubes
- Pancreatin (activity, 4 x USP specifications)
- Bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts)
- Artificial saliva solution
- 0.1 mol/L NaHCO₃ (4.2 g / 500 ml)
- Chelex-100 - Bio-Rad Laboratories
- HCl (0.1 M and 6 M)
- 1.6-cm diameter filtration column (Supleco 20-ml SPE tube, empty fritted)
- Small 1-arm flask to pull vacuum through filtration column
- NaHCO₃ (0.1 mol/L)
- Rocking platform tabletop shaker
- Supplemented DMEM medium
- Supplemented MEM medium
- Cell harvest rinse solution
- 2 ml Transfer pipets
- Continuous Culture Diets
- Fe Solution

Procedure

Seeding

1. Add 2.5 mL complete medium to each plate well then place Transwell insert and add 1.5 mL medium inside each insert.
2. Incubate plate at 37°C for at least 1 hour or even overnight.
3. Remove media from well inserts and add in fresh media (1.5mL) containing ~50,000 cells/cm² to the Transwell inserts.

- a. Insert membrane growth area = 4.67 cm^2
- b. 233,500 cells total ~ 155,666 cells / mL
4. Leave one insert per plate blank, without cells to serve as a control
5. After seeding, return plates to the incubator and examine the cultures periodically and change medium in both compartments every 2 days
6. Allow cells to grow for ~20 days post confluence. Record passage number.
7. Measure and record confluence and tight junction formation using the Phenol Red Flux assay.
 - a. Transwell inserts without IEC-6 cells are treated as a blank.
 - b. Once cells have achieved appropriate Phenol Red Flux (J_{PR}) values and tight junctions have formed, begin experiment.
8. Once cells have grown to full confluence and tight junctions have formed, begin experiment.

Preparation

- Diet Preparation
 - Mix Diet and grind to fit through 1 mm screen.
 - Analyze for iron content
- Minimum Essential Medium (**MEM**)
 - Supplement with:
 - 10 mmol/L PIPES
 - 1% antibiotic
 - hydrocortisone (4mg/L)
 - insulin (5 mg/L)
 - selenium (5 $\mu\text{g/L}$)
 - triiodothyronine (34 $\mu\text{g/L}$)
 - epidermal growth factor (20 $\mu\text{g/L}$)
- Salt Solution
 - Mix together 120 mmol/L NaCl (7.013 g/L) and 5 mmol/L KCl (0.373 g/L)

Day of experiment:

Sample and Digestion Solutions Preparation:

- Complete shortly before use
- All glassware used for sample preparation must be acid washed

- Phosphate Buffer
 - Weigh 2.1 g of Sodium Phosphate Dibasic, Anhydrous and add to a 1 L volumetric flask
 - Add 11.76 g Sodium Phosphate Monobasic, Monohydrate to the flask
 - Add autoclaved DI water until the volume reads 1 L
 - Transfer to a Pyrex jar, add a stir bar and mix well on a stir plate.
 - Check the pH. Target = 6.8 ± 0.1 .
 - Store in the refrigerator until use.

- Peptic digestion buffer
 - 130 mmol/L NaCl (6.604 g/L)
 - 5 mmol/L KCl (0.373 g/L)
 - Adjust to pH 2.0 using 6 N HCl

- Pancreatin Solution (100 mg pancreatin / ml)

*Yields ~60% of the volume of buffer added

 - Weigh 30 g of porcine pancreatin and add 30 ml of phosphate solution.
 - Place on stir plate and mix gently until the pancreatin is dissolved
 - Transfer to 50 ml centrifuge tubes with lids – seal and store until ready to use.
 - Centrifuge in the 50 ml tubes at 1800 x g for 10 min just before use
 - Pour supernatant into clean beaker and store until ready to use, discard particulate material.

- Artificial Saliva Solution
 - Store at 4°C until use
 - Add the following amounts to 500 mL dH₂O and bring to pH 7 with CO₂
 - Na₂HPO₄ - 0.0710 g
 - MgCl₂ x 6 H₂O - 0.1525 g
 - CaCl₂ x 2 H₂O - 0.7350 g
 - KCl - 0.5592 g
 - NaHCO₃ - 2.0163 g
 - NaCl - 0.7500 g
 - α – amylase (1.5 U/mg) - 0.1667 g

- Pepsin Solution
 - Dissolve 0.4 g pepsin in 10 mL of 0.1 mol/L HCl and add to 5 g of Chelex-100
 - Shake on a tabletop shaker for 30 min
 - Pour mixture into filtration column to filter out the Chelex. Set the column into the top of a small 1-arm flask and pull vacuum to help draw liquid through the frit. Chelex should remain on top of the frit, creating a chelex column.
 - Stop pulling the vacuum once foam begins to come through.
 - Add an additional 10 mL of 0.1 mol/L HCl to the column and collect the filtrate into the pepsin solution.

Final total volume of eluted pepsin solution = 17 mL

- Pancreatin/Bile Extract
 - Dissolve 4 ml pancreatin solution and 0.6 g bile extract in 50 mL of 0.1 mol/L NaHCO₃ (4.2005 g NaHCO₃ / 500 ml)
 - Add 25 g Chelex-100 to the mixture and shake for 30 min on a tabletop shaker
 - Pour mixture into filtration column to filter out the Chelex. Set the column into the top of a small 1-arm flask and pull vacuum to help draw liquid through the frit. Chelex should remain on top of the frit, creating a chelex column.
 - Stop pulling the vacuum once foam begins to come through. Pass the collected liquid through the Chelex column one more time.
 - Add an additional 20 mL of 0.1 mol/L NaHCO₃ was to the column and collect the filtrate into the pancreatin/bile solution
 - Final volume = 70 mL

- Fe Solution

(Provides the same Fe concentration as in the 1 g diet sample)

 - Bring 100 mL of sterile distilled water to pH 5.5 using 1 N HCl
 - Add 0.0484 g FeSO₄·7 H₂O to 20 mL of the acidified distilled water & mix well

Treatment Assignments

- Trt 1: Control diet, 0% GSE
- Trt 2: Low diet, 1.33% GSE
- Trt 3: Mid diet, 2.66% GSE
- Trt 4: High diet, 4.00% GSE
- Trt 5: Iron positive control, Fe Soln' + digestion solutions
- Trt 6: Just Digestion solutions
- Trt 7: MEM only (No digestion)
- Blank inserts (without cells) receive treatment 5

Position	Plate 1	Plate 2	Plate 3	Plate 4
A	Blank insert	4	3	7
B	4	5	4	5
C	1	2	Blank insert	1
D	2	3	1	Blank insert
E	6	Blank insert	2	3
F	5	6	7	6

9. Salivary Incubation

- Label 50 mL screw cap culture tubes.
- Weigh 1 g of sample into each tube and record weight.
 - For Treatment 5: 0.5 ml Fe solution and 0.5 ml sterile DI H₂O
 - For Treatment 6: 1 ml sterile DI H₂O
 - For Treatment 7: No digestion
- Add 2 mL artificial saliva, cap tubes and incubate at 37°C for 10 min on the rocking shaker at speed = 7 (55 oscillations/min)

10. Peptic Digestion

- Add 8 mL peptic digestion buffer and adjust pH to 2.0 with 1 N HCl (~1.5 ml)
- Add 0.5 mL of the pepsin solution
- Cap the tube, mix by inversion, place horizontally and incubate at 37°C for 60 min on the rocking shaker at speed = 7 (55 oscillations/min)
- Record the exact timing for each plate – it will be helpful to separate the timing of each plate by ~20 min.

11. Culture preparation:

- Immediately before the intestinal digestion, remove culture medium from both compartments of each well.
- Wash the cell layers 2 times with 37°C PBS at pH 7 and 1 time with supplemented MEM.

- Add 2.0 mL of MEM to the basolateral compartment of the well and 1 ml MEM into the insert on top of the cells.

12. Intestinal Digestion:

- After the 60 min peptic incubation, raise pH of digest to 6 by dropwise addition of 1 mol/L NaHCO₃
- Add 2.5 mL of pancreatin-bile extract mixture and adjust pH to 7 with 1 M NaOH
- Bring volume up to 15 mL with the salt solution prepared earlier
- Vortex for 15 seconds to mix
- Place tubes horizontally on the rocking shaker and incubate for for 120 min at 6 oscillations/min. (Record the exact timing for each plate)

- After 120 min remove tubes from the incubator and vortex for 10 seconds
- Using a 2 ml transfer pipet, transfer ~ 12 ml of digest into the Amicon filtration tubes.
- Ensure all 4 centrifuge cups are balanced and filter membranes are oriented the same way.
- Centrifuge at 4,000 x g for 25 min at 22°C.
- Remove the filter membranes and all remaining particulate.
- Vortex filtrate before pipetting 1.0 ml of each digest solution into the appropriate apical chamber of the well plates.
- Cover the plates and place on the rocking shaker and incubate for for 120 min at 6 oscillations/min.
- Sanitize the plates with ethanol before placing back in the sterile incubator for 22 more hours. (Record the exact timing for each plate)

13. Termination

- After 22 hours, remove plates from the incubator.
- Collect any media or liquid that dialyzed through the insert membrane into the basolateral chamber of the blank wells.
- Remove any media or liquid from the basolateral compartment of all other wells and store in a 2 mL microcentrifuge tube.
 - Keep samples frozen at -20°C until analysis for future iron concentration

Cell Harvest

Materials

- Bench-top sonicator
- Deionized water
- Cell scrapes
- Tubes to collect cells
- Rinse solution
- PBS

Solution recipe:

- Rinse Solution (140 mM NaCl, 5 mM KCl, 10 mM PIPES)
 - Using acid washed tools, weigh the chemicals into a sterile, acid washed 2 L flask:
 - 8.1816 g NaCl
 - 0.3727 g KCl / L Sterile DI H₂O
 - 3.0237 g PIPES / L Sterile DI H₂O
 - Check pH (should be 7)
 - Add 1 L sterile DI H₂O
 - Mix using a sterile, acid washed stir bar
 - Pre-warmed to 37°C

Procedure

9. Harvest cells 24 h after applying the digest to the cell monolayer.
10. Remove all media or liquid covering the cells by decanting into a waste container.
11. Wash cells with a 2 mL volume of cold PBS solution.
12. Decant PBS.
13. Add 2 mL of rinse solution, swirl to wash then decant liquid off. Repeat 1x.
14. Add 2 mL of DI water and place plate on a rack such that the bottom of each plate is in contact with the water of a bench top sonicator – kept in a cold room at 4°C
15. Sonicate cells for 15 minutes
16. Using a cell scraper, scrape the cells from the insert and harvest along with the 2 mL volume of water in each well and store at -20°C until analysis for cellular protein and ferritin formation

Cell Culture Analysis

All glassware used in sample preparation and analysis must be acid-washed.

Cellular Protein Concentration

1. Label 2, 2 ml microtubes per sample.
2. Using a 1 ml pipet, mix cell lysate in the tube by pipetting up and down 5 times.
3. Vortex for 15 seconds.
4. Immediately pipet 500 μ L into each new tube.
5. Solubilize cellular protein by pipetting 20 μ L of 0.5 M NaOH into each tube.
6. Vortex for 10 seconds.
7. Measure protein concentration using the semi-micro adaptation of the Bio-Rad DCTM protein assay kit (Bio-Rad Laboratories, Inc., Hercules, California).
 - a. Standards:
 - i. Dilute NaOH – (4% NaOH, 96% DI H₂O)
Mix 0 mg/ml – mix 160 μ L 0.5 M NaOH and 4000 μ L DI H₂O
 - ii. 1 mg/ml - Dilute 1 ml of standard BSA solution (2mg/ml) with 1 ml of dilute NaOH solution.
 - iii. Serial dilute the 1 mg/ml sample with dilute NaOH solution to achieve standards with concentrations of:
0.5, 0.25, 0.125, & 0.0625 mg protein/ml
 - iv. Create a 0 standard using just the dilute NaOH solution.
 - b. Read the absorbance on a microplate reader fitting the standard curve as a 2nd order polynomial.

Ferritin Analysis

Materials and Equipment Needed:

- Ferritin Kit (Rat Ferritin ELISA, Immunology Consultants Laboratory, Inc., Portland, Oregon)
- Pipette (2 μ L to 200 μ L)
- Test Tubes
- Microtitre washer / aspirator
- Deionized H₂O
- Microtitre Plate reader
- Timer
- Glassware for preparation of reagents and buffer solutions

Procedure

- Ferritin content measured by immunoradiometric assay
- Using a 1 ml pipet, mix the sonicated cell lysate in the tube by pipetting up and down 5 times.
- Vortex for 15 seconds.
- Samples are diluted in a 1:5 ratio (cell lysate:diluent)
- The ratio of ferritin/total protein (expressed as ng ferritin/mg protein) is used as an index of iron bioavailability.

Iron Analysis

Reagents

- 0.5% Triton X-100- Add 500 ul into 100ml of dH₂O. Mix well.
- 2N HCl- 200ml of dH₂O into a 1L volumetric flask. Add 166mL of concentrated HCL (Fisher A508-P212) then dilute to volume with dH₂O. Rinse all glass ware with 2N HCl
- Iron reference standard 1000 ppm (Fisher Cat # SI124-100)

Standards

Make 100ppm Fe standard- Add 10ml of 1000ppm reference standard to 100ml volumetric flask and make up to 100ml with dH₂O.

Make the following working standards up to 100ml in a volumetric flask with 2N HCl.

Fe working standards	Fe reference standard into volumetric flask
2 ppm	2 ml of 100 ppm Fe STD
5 ppm	5 ml of 100 ppm Fe STD
10 ppm	10 ml of 100 ppm Fe STD

Make the following final standards using the working standards and 0.5% triton

Fe standards	Fe working standard into 15ml tube	0.5% triton
0.2 ppm	1 ml of 2 ppm Fe working STD	9ml
0.5 ppm	1 ml of 5 ppm Fe working STD	9ml
1 ppm	1 ml of 10 ppm Fe working STD	9ml
2 ppm	2 ml of 10 ppm Fe working STD	8ml

Procedure

Pipette 1ml of sample into a plastic tube and add 1ml of 0.5% Triton X-100. Vortex vigorously.

Prepare a blank by pipetting 1ml distilled water with 1ml of 0.5% Triton X-100.

Samples are now ready for analysis on the AA spec.

Preliminary Cell Culture Experiment Results

Statistics

The GLM procedure of SAS was used to analyze the effect of treatment. Means separations

Experiment 1 – Iron and Grape Seed Extract Solutions (No Digestion)

The amount of ferritin produced was standardized per unit of cellular protein. Treatment significantly affected the ng ferritin produced per mg of cellular protein ($P = 0.006$).

Table A-1. Cell culture experiment one treatment descriptions.

Treatment Number	Description
1	Iron solution
2	Low (1.33%) ¹ GSE solution + Iron solution
3	Mid (2.66%) ¹ GSE solution + Iron Solution
4	High (4.00%) ¹ GSE solution + Iron Solution
5	Tannic Acid (1.33%) ¹ solution + Iron solution
6	High (4.00%) ¹ GSE solution only
7	Minimum Essential Media only

¹GSE and Tannic Acid were solubilized in DI H₂O. Percentages are displayed as wt/vol.

Table A-2. Ferritin formation when IEC-6 cells were exposed to iron, tannin, and control solutions.

Treatment Number	ng ferritin/mg cellular protein	SEM
1	634.89 ^c	99.690
2	1338.53 ^a	128.700
3	705.03 ^{bc}	111.457
4	1017.95 ^{ab}	111.457
5	874.97 ^{bc}	128.699
6	750.07 ^{bc}	128.699
7	594.17 ^c	128.699

When only the iron and GSE treatments were compared in the absence of any control treatments (i.e. comparing treatment numbers 1-4), treatment was still a significant effect on ng ferritin per mg cellular protein ($P = 0.01$).

Table A-3. Ferritin formation when IEC-6 cells were exposed to iron and grape seed extract solutions.

Treatment Number	ng ferritin/mg cellular protein	SEM
1	634.89 ^c	114.047
2	1338.53 ^a	147.234
3	705.03 ^{bc}	127.509
4	1017.95 ^{ab}	127.509

Experiment 2 – Digested Continuous Culture Diets

The amount of ferritin produced was standardized per unit of cellular protein. Treatment significantly affected the ng ferritin produced per mg of cellular protein ($P = 0.04$). The iron solution treatment (Treatment 5) resulted in the greatest ferritin production per unit of cellular protein.

Table A-4. Cell culture experiment two treatment descriptions

Treatment Number	Description
1	Control Diet - 0% GSE ^{1,2}
2	Low Diet - 1.33% GSE ^{1,2}
3	Mid Diet - 2.66% GSE ^{1,2}
4	High Diet - 4.00% GSE ^{1,2}
5	Iron solution (formulated to have same Fe concentration as continuous culture Diets) ²
6	H ₂ O only ²
7	MEM only (No digestion)

¹GSE and Tannic Acid were solubilized in DI H₂O. Percentages are displayed as wt/vol.

²Treatments 1-6 were put through a simulated salivary incubation, peptic digestion, and intestinal digestion.

Table A-5. Ferritin formation when IEC-6 cells were exposed to *in vitro* intestinally digested continuous culture diets.

Treatment Number	ng ferritin/mg cellular protein	SEM
1	652.93 ^b	741.423
2	651.68 ^b	741.423
3	640.67 ^b	1048.531
4	502.29 ^b	741.423
5	5057.66 ^a	1048.531
6	564.55 ^b	1284.183
7	424.53 ^b	1284.183

When only the four continuous culture diets were compared in the absence of any control treatments (i.e. comparing treatment numbers 1-4), treatment was no longer a significant effect on ng ferritin per mg cellular protein ($P = 0.22$).

Discussion of Results and Experiment Limitations

Although GSE was determined to be an effective iron chelator using the iron-binding capacity method described in Chapter 2, high variation observed in both cell culture experiments precluded valuable results interpretation. We hypothesize that methodology issues resulted in the extensive variation observed. The initial methods by Glahn and others (1998, 2002) utilized a dialysis membrane with a 12kD molecular weight cutoff (Spectra/Por 2 Dialysis Discs, Spectrum Laboratories, Inc., Rancho Dominguez, CA). We first attempted using this product instead of the Amicon centrifuge filtrations devices, however, water, cell culture media, or any experimental solution would not successfully dialyze through the membrane. Centrifugation through the Amicon filters successfully dialyzed the solution, but did not consistently inhibit enzymes from passing through when used in the digestion experiment. Therefore, when the filtrate was applied to the cell monolayers, enzymatic digestion of some of the experimental

units occurred. Preliminary results indicate that successful results could be obtained if this problematic step in the methodology is improved.