PHYSICALLY EFFECTIVE FIBER THRESHOLD, APPARENT DIGESTIBILITY, AND NOVEL FECAL MICROBIOME IDENTIFICATION OF THE LEOPARD TORTOISE (*STIGMOCHELYS PARDALIS*)

A Thesis
presented to
the Faculty of California Polytechnic State University,
San Luis Obispo

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Agriculture

by
Breanna Paige Modica
September 2016

COMMITTEE MEMBERSHIP

TITLE: Physically Effective Fiber Threshold, Apparent Digestibility, and Novel Fecal Microbiome Identification of the Leopard Tortoise (*Stigmochelys pardalis*)

AUTHOR: Breanna Paige Modica

DATE SUBMITTED: September 2016

COMMITTEE CHAIR: Mark S. Edwards, Ph.D.
Associate Department Head, Animal Science

COMMITTEE MEMBER: Daniel G. Peterson, Ph.D.
Professor of Animal Science

COMMITTEE MEMBER: Christopher L. Kitts, Ph.D.
Department Chair, Biology

ABSTRACT

Physically Effective Fiber Threshold, Apparent Digestibility, and Novel Fecal Microbiome Identification of the Leopard Tortoise (*Stigmochelys pardalis*)

Breanna Paige Modica

Particle size distribution of diet, feces, and change from diet to feces, as well as apparent digestibility (aDig, %) of selected nutrients, and novel fecal microbiome identification of mature female leopard tortoises (*Stigmochelys pardalis*, n = 16) fed exclusively one of three, nutritionally complete, pelleted diets were evaluated in a blind, complete randomized design study. Two diets included insoluble fiber (powdered cellulose) consisting of either 2.0 mm or 0.2 mm length. Insoluble fiber provides nutritional and physical benefits to both the animal host and the microorganisms that inhabit the gastrointestinal tract. Insoluble fiber length was used as a means of evaluating a physically effective fiber (peNDF) definition for hindgut-fermenting vertebrates. Numerical trends of each diet particle size distribution indicated a greater amount of particle recovery on the 2.0 mm sieve for the 2.0 mm diet, and a greater particle recovery on the 0.125 mm sieve for the 0.2 mm diet, both as expected based on the added fiber lengths. Fecal particle size distributions were not different between diets, however, distributions of the change in particle size from diet to feces were different between diets. Similar fecal particle size distributions across diet suggests both cellulose lengths are below the peNDF threshold of the leopard tortoise. Apparent digestibility (aDig, %) of dry matter (DM) and organic matter (OM) was not different based on diet, method, or a diet and method interaction; aDig (%) of neutral detergent fiber (aNDF) and sequential acid detergent fiber (sADF) was different based only on diet. These results suggest that while aDig (%) of OM did not

change, the source of OM digestibility shifted, as both aNDF and sADF digestibility increased with the cellulose-added diets compared to the control diet. An increase in insoluble fiber digestibility suggests an “effectiveness” of the cellulose lengths. At both bacterial phyla and genera levels, fecal microbiomes were more similar between tortoises fed the cellulose-added diets versus the control diet, suggesting that the hindgut microbial communities adjusted in the hindgut of tortoises fed the cellulose-added diets by shifting proportions of microbes, based on their role in the hindgut (i.e., cellulose digestion), to accommodate for the addition of cellulose in the two treatment diets. This may explain the similarity among fecal particle size distributions, and suggests that adaptability of the hindgut microbial communities should be considered when defining peNDF for hindgut-fermenting vertebrates.

ACKNOWLEDGMENTS

First, I need to acknowledge the opportunity provided to me by my committee chair, Dr. Mark Edwards. Without the support and guidance that he provided me during my undergraduate career, I know that I would not be in the position I am today. When I first introduced myself to Dr. Edwards as a new undergraduate animal science student, I had no idea that we would develop a professional relationship that would take me through two animal science degrees, and a multitude of research projects. Dr. Edwards is not only an example of professionalism, but he is also the epitome of modesty. I will always be grateful for the lessons he has taught me; most importantly, that working towards a solution for the good of the group – whether devising the solution yourself or setting the stage for others to continue or finalize the work – is the greatest contribution a researcher can make.

I must extend my sincerest gratitude to my committee members, Dr. Dan Peterson and Dr. Chris Kitts, for the endless emails and meetings that kept me on track. Their support of this research was absolutely integral to its completion. Without Dr. Peterson's support, I would not have developed the stamina that kept me on schedule with each facet of this research. And without Dr. Kitts, I would not have been able to learn an entirely new aspect of animal nutrition in the form of the microbiome.

It is beyond necessary to thank fellow graduate students Emily Schwartz and Rhianne Maxwell for their help with this project. They were involved in so many aspects, from the beginning stages of planning to sample collection and analyses. It is safe to say that this work would not have been completed without them. The help of students in the

Reptile Husbandry Enterprise (2015 – 2016) and Nutrition Research Enterprise (2016) also played major roles, and had a huge impact on this research.

I would like to thank JJ Rettenmaier USA LP for their generous donation of the cellulose used in the research diets, and Land O'Lakes, Inc. and Dr. Troy Tollefson for the integral donation of Mazuri® Tortoise Diet, for pelleting all of the diets used in this research, and for donating the finished products. I am also extremely grateful for the grants that supported this research. Contributors include the East Texas Herpetological Society and California Polytechnic State University, San Luis Obispo.

Lastly, I need to thank my husband, Greg Modica, for years of support and encouragement. He has been by my side through four undergraduate institutions, countless hours of coursework and research, endless time spent with the leopard tortoises, and so much more. I would also like to thank the wonderful staff at the Charles Paddock Zoo for teaching me to appreciate all facets of animal husbandry and for setting me on the path to studying comparative nutrition.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

aDig, %	Apparent digestibility, the percentage of a feed, or nutrient, that is acted on in the digestive tract to a point that it can be absorbed, without considering endogenous losses, calculated as the amount of nutrient ingested minus the amount of nutrient present in feces, as a percentage of the amount of nutrient ingested
ADF	Acid detergent fiber, comprised of cellulose and lignin
AIA	Acid insoluble ash, inorganic (mineral) content insoluble in 2M HCl, determined after determination of initial ash gravimetrically
aNDF	Neutral detergent fiber treated with amylase, comprised of cellulose, hemicellulose and lignin
CF	Crude fiber, the amount of indigestible plant components in food
CV	Coefficient of variation, standardized measure of variation of a distribution in the form of a ratio of the standard deviation to the mean
DM	Dry matter, the non-water portion of a sample
DMB	Dry matter basis
dMEAN	Discrete mean (particle size), calculated as the sum of the equation $dMEAN = \sum_{i=1}^{n} p(i) * \frac{S(i+1) + S(i)}{2}$ where S is sieve pore size, $S(i + 1)$ is the sieve pore size smaller than $S(i)$, the preceding sieve pore size; $p(i)$ is the proportion of particles retained on a given sieve
GE	Gross energy, the total potential energy content of a sample
ME	Metabolizable energy, available food energy after accounting for gaseous, fecal, and urinary losses
MPS	Mean particle size, the average particle size retained on a given set of sieves
NDF	Neutral detergent fiber, comprised of cellulose, hemicellulose, and lignin
OM	Organic matter

OTU	Operational taxonomic unit, cluster of 16s rRNA sequences classified based on similarity with other sequences within a data set
peNDF	Physically effective neutral detergent fiber, defined as the combined influence of fiber particle size and NDF content that promotes hindgut motility as it relates to microbial fermentation of structural carbohydrates
sADF	Sequential acid detergent fiber, determined after NDF determination using the same sample
SCFA	Short chain fatty acid, the end-product of insoluble fiber fermentation that supplies energy to the host
SD	Standard deviation, the amount of variation in a data set
SEM	Standard error of the mean, the standard deviation of a distribution statistic (i.e., mean)

1. INTRODUCTION

The leopard tortoise (*Stigmochelys pardalis*) is a solitary, terrestrial reptile inhabiting central and south eastern Africa. Its geographical range is widest of all African tortoise species, encompassing arid and mesic environments (McMaster and Downs, 2006a). Due to its substantial geographic distribution, absolute home range definition has not yet been established (Hailey and Coulson, 1996; McMaster and Downs, 2009). *Stigmochelys pardalis* is considered a generalist herbivore, consuming mainly grasses and succulents, but ingesting other available food items such as herbs and fruits (Rall and Fairall, 1993; Hailey, 1997; Kabigumila, 2001a; McMaster and Downs, 2008). Food items are ingested in proportion to abundance, and dietary variety increases seasonally (Rall and Fairall, 1993; McMaster and Downs, 2008).

Digestive anatomy and physiology of herbivorous turtles and tortoises has been described for several species, including *S. pardalis*. The digestive tract includes a horny beak, stomach, small intestine, and large intestine ending at the cloaca (Barboza, 1995; Taylor et al., 1996; Stevens and Hume, 2004; Schwenk and Rubega, 2005). The leopard tortoise hindgut consists of the large intestine, which features a small cecal dilatation and well-defined colon (Taylor et al., 1996; Hailey, 1997). The hindgut hosts substantial concentrations of microbes that digest and ferment structural carbohydrates, which comprise a large portion of plant-based diets (Wrong et al., 1981; Stevens and Hume, 2004). Structural carbohydrates including cellulose, hemicellulose, and lignin, are measured as neutral detergent fiber (NDF). While lignin is indigestible, cellulose and hemicellulose are digested only by microbes. Without these microbes, cellulose and hemicellulose would be unavailable for nutritional contribution to the animal (host).

Microbes digest structural carbohydrates (fiber) into smaller components, and further ferment these components by chemically converting them into other compounds, such as short chain fatty acids. Both processes occur simultaneously in the hindgut. Microbes, unlike the animal itself, produce enzymes capable of cleaving the ß-1,4 glycosidic linkages found in structural carbohydrates into short-chain carbohydrates (Bayer et al., 1998; Schwarz, 2001; Carere et al., 2008). These digested carbohydrates are transported into the microbial cell cytosol where they are fermented into short-chain fatty acids (SCFA) (Carere et al., 2008). Short-chain fatty acids are then transported out of the microbial cell, and absorbed across the large intestinal mucosa (Wrong et al., 1981). Aside from structural carbohydrates, dietary components that reach the large intestine supply microbes with nutrients and energy (Leser and Mølbak, 2009).

Captive tortoise diets have traditionally varied in composition, containing domestic produce, hay, and commercial dog and cat foods (Davis, 1979; Donoghue and McKeown, 1999; Lickel, 2010; Ritz et al., 2012). These diets pose two concerns, 1) nutrient imbalance, which may result in nutritional disorders (Donoghue and McKeown, 1999; Fledelius et al., 2005; Ritz et al., 2012), and 2) most extant herbivorous reptiles lack teeth (Spellerberg, 1982; Norman and Weishampel, 1985; Stevens and Hume, 1998), and may be adapted to larger ingesta particles than what these diets provide.

Physically effective neutral detergent fiber (peNDF) accounts for fiber particle length and NDF content as a measure of physical fiber characteristics (Mertens, 1997). Physical fiber characteristics are related to digestibility and digesta passage, among other parameters (Bjorndal et al., 1990; Mertens, 1997). In contrast to “nutritionally effective” fiber that provides the animal with nutrients, peNDF provides bulk in the diet, resulting in

slower digesta passage through the gastrointestinal tract so that nutritionally effective fiber may be adequately digested and fermented by microbes (Van Soest, 1994). Characteristics of peNDF are considered when formulating ruminant diets (Yang and Beauchemin, 2007), whom rely heavily on rumination and ruminal microbial digestion and fermentation of structural carbohydrates for nutrient availability. Fiber particle length and NDF content (peNDF) may also be important when formulating hindgut-fermenter diets, as they also rely heavily on microbial digestion and fermentation. Ingesta fiber particle length and recovered fecal particle length were investigated as a method of testing a hypothesized definition of peNDF, using an herbivorous tortoise species as a model for other hindgut-fermenting vertebrates. Apparent digestibility (aDig, %) of dry matter (DM), organic matter (OM), neutral detergent fiber (aNDF), and sequentially determined acid detergent fiber (sADF) was also determined with regards to peNDF. In a novel investigation, the microbial communities of 16 *S. pardalis* individuals fed one of three diets differing in fiber particle length were also identified as baseline data for identifying gastrointestinal microbial deviations based on dietary management.

2. LITERATURE REVIEW

2.1 Natural History and Physiology

The leopard tortoise (*Stigmochelys pardalis*) is characterized by a shell, lack of temporal fenestrae, and lack of a nuchal shield (Porter, 1972; Spellerberg, 1982; Patterson, 1987; Boycott and Bourquin, 1988; Fritz and Havaš, 2007). Terrapins, turtles, and tortoises are in the order Testudinea, often referred to as chelonians (Stevens and Hume, 1998, 2004). *Stigmochelys pardalis* was formerly described as having two subspecies, identified as *Geochelone pardalis pardalis* from southern parts of Africa, and *Geochelone pardalis babcocki* from central Africa (Le et al., 2006; Jessop, 2009). A recent study proposes that there are no subspecies of *S. pardalis,* but rather phylogeographical differences in tortoises inhabiting different regions of Africa (Fritz et al., 2010b). This was previously predicted by Boycott and Bourqin (1988).

South Africa is home to a vast diversity of terrestrial tortoises, housing nearly one third of the world's 42 extant species (Patterson, 1987; McMaster and Downs, 2006a; Fritz et al., 2010a). *Stigmochelys pardalis* is the second largest of 13 southern African tortoise species, native to many areas of central and southern Africa including Angola, the Cape, Nama-Karoo, Namibia, Sudan, and Tanzania (Rall and Fairall, 1993; Kabigumila, 2000, 2001a; McMaster and Downs, 2008, 2013a; Fritz et al., 2010a). *Stigmochelys pardalis* is solitary and occupies a large home range (Hailey and Coulson, 1996; Wimberger et al., 2011), although there are confounding variables such as regional climate, mineral availability, and gender that make home range difficult to absolutely define (McMaster and Downs, 2009).

Tortoise shells are characterized into two main portions, 1) the dorsal portion of the shell, called the carapace, and 2) the ventral portion of the shell, called the plastron (Patterson, 1987). The entire shell is subdivided into many smaller portions called scutes, each referenced by its anatomical location (i.e., supracaudal scute) (Patterson, 1987). The carapace of *S. pardalis* adults is dull yellow, tinged with black markings (Patterson, 1987), while the plastron is typically only dull yellow in color. The straight carapace length of wild adult *S. pardalis* can reach up to 60 cm (Patterson, 1987). Wild adult *S. pardalis* body mass averages 10 to 15 kg, with captive animals reported as exceeding 40 kg (Patterson, 1987).

Stigmochelys pardalis is listed by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) as Appendix II: not directly threatened by extinction, but under controlled trade to manage survival (CITES, 2014a). Aside from habitat loss, the exotic animal trade is a large threat to *S. pardalis*. As of 2014, there have been suspensions in the exportation of wild *S. pardalis* specimens from multiple South African countries including The Democratic Republic of the Congo, Mozambique, and Uganda (CITES, 2014b). In a recent study compiling information from world-wide exotic animal trade databases and reports (2006–2012), Testudinae was reported 17 times more often than other reptile orders in the exotic pet trade (Bush et al., 2014). Exotic animals pose unique concerns in captivity, as there are still large gaps in our knowledge of their basic natural propensities.

Stigmochelys pardalis is poikilothermic, ectothermic, and diurnal; it is able to survive over a wide range of body temperatures by influencing the rate of heat exchange between itself and the environment (Spellerberg, 1982), and has its active period from

sunrise to sunset (McMaster and Downs, 2013a). Field core body temperature of *S. pardalis* ranges from 25.0° to 35.0°C in summer (n = 4), and 8.0° to 30.0°C in winter (n = 5) (McMaster and Downs, 2013b). In the same study, mean cloacal temperature was recorded as 24.7°C during summer and 11.9°C during winter (McMaster and Downs, 2013b). Voluntary core body temperature range of several reptilian families, including Testudinidae, has been reported as 8.0° to 42.3°C (Porter, 1972), with 15.0°C representing mean voluntary minimum core body temperature and 39.0°C representing mean voluntary maximum core body temperature (Spellerberg, 1982).

Reptiles use a range of mechanisms to regulate body temperature including posture, behavior and utilization of microhabitats (Spellerberg, 1982; McMaster and Downs, 2013b). *Stigmochelys pardalis* will seek shelter in the form of bushes, undergrowth, and preformed burrows (Grobler, 1982; Kabigumila, 2001d; McMaster and Downs, 2006b). It has been demonstrated that wild *S. pardalis* will passively thermoregulate, both in warm and cold seasons, by orienting themselves to minimize or maximize the amount of sun exposure to their shell (McMaster and Downs, 2006b). Passive thermoregulation costs less energy than active thermoregulation, which generates heat from the breakdown of ingested food (Shine, 2005).

Thermal biology is an important aspect for ectothermic digestion, in which there has been a demonstrated relationship between gastric juice secretion and ambient temperature. Although a relationship between temperature and digestion has not been investigated in *S. pardalis*, Sadeghayobi et al. (2011) demonstrated a relationship between ambient temperature and retention time of indigestible particles (1.0 mm, 1.8 mm, 3.5 mm, 5.0 mm, 5.5 mm, 10.0 mm) and seeds (guava, papaya, passion) with 19 Galapagos tortoises

(*Chelonoidis nigra*) in semi-natural conditions. Retention time was inversely correlated with ambient temperature, increasing as the temperature decreased (Sadeghayobi et al., 2012).

Digesta passage rate is directly related to ambient temperature for ectotherms. It is measured by feeding the animal indigestible and inert markers that can be fully recovered in the feces (Schneider and Flatt, 1975). Digesta passage rate can be measured as time to first marker appearance in the feces (transit time; TT_1), time to 50% marker recovery in the feces (T_{50}), time to maximum marker recovery in the feces (T_{MAX}), and retention time (R_{GIT}) (Stevens and Hume, 1998). Retention time is the average amount of time a digestive marker spends in the gut (Stevens and Hume, 1998). Digesta passage rate for reptiles is longer than birds or mammals, typically spanning several days (Barboza, 1995; Stevens and Hume, 2004; Tracy et al., 2006). With mean 24 h ambient temperature ranging from 16.2°C to 29.8°C, mean digesta passage rate to maximum recovery of chromium (Cr)-mordanted neutral detergent fiber (NDF) of 18 juvenile female *S. pardalis* fed a nutritionally complete, herbivorous tortoise diet (Mazuri® No. 5M21, fed 7 d per week for 35 d) was 12.3 d (Lickel, 2010). At a constant 30°C ambient temperature, Hailey (1997) demonstrated retention time of 6 hinge-back tortoises (*Kinixys spekii*) and 6 *S. pardalis* individuals to be 2.2 d and 3.8 d, respectively, when fed looped polyester thread markers (2.0 cm circumference) with kale leaves.

Particulate retention time is of specific importance for structural carbohydrates (fiber), which require sufficient exposure duration to microbes for attachment, digestion, and fermentation. Therefore, particle size may affect retention time. Mean retention time of red-foot tortoises (*Geochelone carbonaria*, $n = 4$) increased as particle size (2.2 diameter

polyethylene tubing) increased, with 2.0 mm length particles being retained for 170 h, 5.0 mm length particles being retained for 185 h, and 10.0 mm length particles being retained for 363 h (Guard, 1980). The potential benefit of increased larger particle retention, is larger particles require more time for digestion than smaller particles (Bjorndal et al., 1990).

Stigmochelys pardalis is a generalist herbivore, consuming mainly grasses and succulents, but ingesting other available plant food items such as herbs and fruits (Patterson, 1987; Rall and Fairall, 1993; Hailey, 1997; Kabigumila, 2001a, d; McMaster and Downs, 2008). Succulent forbs comprise most of the diet in central Africa (Kabigumila, 2001a), while grasses comprise most of the diet in southern Africa (Rall and Fairall, 1993). Observations of wild *S. pardalis* demonstrate a preference for shorter over taller grasses, possibly due to the inability of *S. pardalis* to further masticate food after initial cropping (Kabigumila, 2001a). Ingestion has been demonstrated as proportional to plant abundance for herbivorous African tortoises (Rall and Fairall, 1993; Joshua et al., 2010), which varies between regions due to temporal differences (McMaster and Downs, 2008).

Ingestion of non-plant material such as bone, carrion, feces, and soil has also been reported (Skoczylas, 1978; Marlow and Tollestrup, 1982; Patterson, 1987; Milton, 1992; Hailey, 1997; Kabigumila, 2001a). An observational study of wild *S. pardalis* reported 97.8% ingestion of plant items and 2.2% ingestion of inorganic matter out of a total 124 feeding observations (Kabigumila, 2001a). This is consistent with other reports that non-plant inorganic matter generally comprises less than 5% of herbivorous tortoise diets (Hailey, 1997). Such behavior may be a strategy to meet mineral requirements necessary

for shell development and egg production (Hailey, 1997; Fledelius et al., 2005; Simang et al., 2010).

Plant-water content and free-standing water are important hydration sources for many tortoise species. Accessibility of these sources can be greatly affected by regional climate (McMaster and Downs, 2008). The sympatric angulate tortoise (*Chersina angulata*) has been observed ingesting condensation-covered leaves in the Western Cape Province of South Africa (Joshua et al., 2010). Although rare observations (0.9%), Kabigumila (2001b) observed adult and subadult wild *S. pardalis* drinking water from ponds in northern Tanzania, while McMaster and Downs (2006a) observed wild *S. pardalis* drinking from livestock watering holes in the Nama-Karoo.

2.2 Gastrointestinal Tract Anatomy and Physiology

2.2.1 Headgut

The mouthparts of *S. pardalis* are characterized by an outer keratinized beak with an internal hard palate and lack of teeth. Chelonian embryos possess partial dental lamina tissue that disappears during early development (Spellerberg, 1982). Some chelonians, including *S. pardalis*, have serrated tooth-like structures on the outer edges of the internal hard palate, although further research is needed to identify origin and function (Spellerberg, 1982). Without teeth, chelonians do not have the ability to masticate, and therefore, cannot mechanically reduce ingested food particle size (Bjorndal, 1997; Fritz et al., 2010b).

Chelonian upper and lower jaws are equivalent in size and shape. The upper jaw is attached to the base of the skull and immobile (Spellerberg, 1982), allowing only a scissor-like movement, cropping food with the lower jaw opening and closing (Stevens and Hume, 2004). A well-developed hyoid apparatus is present, supporting movement of the tongue,

and the larynx (Porter, 1972). Salivary glands are also present, secreting only mucus (Stevens and Hume, 2004). An anatomical study of *Xerobates agassizzi*, an herbivorous desert-dwelling tortoise, revealed cornified epithelia and mucous glands lining the tongue and esophagus (Barboza, 1995). Keratinization and cornification of mouthparts may allow for ingestion of abrasive plant material with minimal damage to the headgut, while mucous secretions lubricate the bolus for deglutition.

2.2.2 Foregut

The esophagus is a simple tube, easily distinguished from the stomach (Spellerberg, 1982). Palatopharyngeal folds are present, and pull medially to create an opening for food to pass through during deglutition (Stevens and Hume, 2004). The esophageal walls are comprised of folded, longitudinal pleats that increase in diameter when unfolded (Porter, 1972). Both inner and outer layers of smooth muscle are present, arranged circularly and longitudinally, respectively (Stevens and Hume, 2004). For the herbivorous Russian tortoise (*Testudo horsfieldii*), it has been suggested that these muscles help to mechanically digest food (Skoczylas, 1978). At the terminal end of the esophagus, the inner circular muscle layer creates a well-defined sphincter that is absent in most mammals (Stevens and Hume, 2004). This may be analogous to the cardiac sphincter found in the equine foregut.

The chelonian stomach is a highly muscular, "L"-shaped organ (Barboza, 1995). In *S. pardalis*, it is located on the left ventral side near the left lobe of the liver (Taylor et al, 1996). It is subdivided into two types of epithelium, 1) fundic glandular and 2) pyloric glandular regions (Guard, 1980; Hailey, 1997; Stevens and Hume, 2004). The fundic region secretes hydrochloric acid (HCl) and pepsinogen to acidify chyme and initiate protein digestion, while the pyloric region secretes only mucus (Guard, 1980). It has been

suggested that the gastric juice of herbivorous reptiles acts as a bacteriocide, keeping cellulolytic microbes from inhabiting the stomach and initiating fermentation in the foregut (Skoczylas, 1978). Fasting stomach pH of the herbivorous Greek tortoise (*Testudo graeca*) has been reported as 7.5 to 8.0 (Skoczylas, 1978). Additionally, the rate of gastric juice secretion in *T. graeca* after histamine injection was 0.75 to 1.0 ml/h^{-1} (Skoczylas, 1978). After barium sulfate administration, complete gastric emptying of captive adult *S. pardalis* (3 males, 3 females) occurred between 5 and 9 h with an average emptying time of 6.2 h (Taylor et al., 1996). Average wet mass of the stomach of 4 adult *S. pardalis* specimens has been reported as 3.0% of total body mass and 24.2% of total gut wet mass contents (Hailey, 1997). The stomach meets the small intestine via the pyloric sphincter (Barboza, 1995).

2.2.3 Midgut

The small intestine of *S. pardalis* is convoluted (Taylor et al., 1996), but less so than mammals (Guard, 1980). It is shortest in length in herbivorous reptiles (Guard, 1980), and is divided into the duodenum, jejunum, and ileum. The main function is to digest nutrients in chyme as it moves from the stomach to the hindgut. At the pyloric sphincter, pancreatic and bile ducts enter the small intestine (Taylor et al., 1996; Diaz-Figueroa and Mitchell, 2006).

The pancreas, an exocrine gland, secretes amylolytic, lipolytic and proteolytic enzymes into the small intestine for digestion of soluble carbohydrates, fats and proteins (Skoczylas, 1978; Stevens and Hume, 2004). The pancreas adjusts enzymatic composition to accommodate digestion of varying dietary components (Spellerberg, 1982). Pancreatic secretion is also alkaline, aiding in neutralizing acidic chyme from the stomach (Skoczylas,

1978). The liver produces bile, which is stored in the gallbladder in turtles, crocodilians, and most lizards, until the midgut accumulates high concentrations of lipids (Stevens and Hume, 2004; Diaz-Figueroa and Mitchell, 2006). However, the gallbladder is absent in some hindgut-fermenting vertebrates (Schwartz, 2015), possibly due to the adaptation to grazing of low-fat (plant) material. Continual secretion of bile directly into the small intestine has been demonstrated for the domestic horse (*Equus caballus*) (NRC, 2007). Bile works with lipases secreted from the pancreas to digest triglycerides into diglycerides and monoglycerides (Skoczylas, 1978; Stevens and Hume, 2004).

The small intestinal mucosa of chelonians is wavy in appearance (Taylor et al., 1996), with long villi that decrease in length toward the ileum (Barboza, 1995). Mucosa and villi aid in moving chyme from the stomach to the hindgut (Stevens and Hume, 2004). Barium sulfate first entered the small intestine of 3 male and 3 female captive adult *S. pardalis* between 0.2 and 1 h, and emptied between 9 and 15 h, with an average of 10.8 h (Taylor et al., 1996). Average wet mass of the small intestine of 4 adult *S. pardalis* specimens has been reported as 2.0% of total body mass and 13.5% of total gut wet mass contents (Hailey, 1997). The small intestine meets the hindgut at the ileocolonic sphincter (Guard, 1980; Stevens and Hume, 2004).

2.2.4 Hindgut

The large intestine is divided into the cecum and colon, and ends at the cloaca. The cecum and ileocolonic sphincter signify the transition from midgut to hindgut. The cecum of *S. pardalis* (Taylor et al., 1996), red-foot tortoises (*Geochelone carbonaria*) and yellow-foot tortoises (*G. denticulata*) (Moskovits and Bjorndal, 1990) has been recognized as a

small dilatation at the ileocolonic sphincter (Stevens and Hume, 2004). Following the cecal dilatation is the proximal colon preceding the distal colon.

Antiperistalsis is speculated to be an important mechanism of the large intestine for tortoises due to the lack of compartmentalization (Guard, 1980; Taylor et al., 1996). Antiperistalsis propels larger food particles back toward the proximal colon for longer periods of exposure to microbial digestion and fermentation (Guard, 1980). Prolonged digesta retention has been evidenced by Taylor et al. (1996) when barium sulfate first entered the large intestine of captive adult *S. pardalis* (3 males, 3 females) between 5 and 8 h, and emptied between 144 and 166 h with an average of 153 h. Average wet mass of the large intestine of 4 adult *S. pardalis* specimens has been reported as 8.4% of total body mass and 62.3% of total gut wet mass contents (Hailey, 1997). Average hindgut pH of 6.5 has been reported for the green sea turtle (*Chelonia mydas*) (Bjorndal et al., 1991b), and 7.5 for the herbivorous green iguana (*Iguana iguana*) (Troyer, 1984). Yokoyama and Johnson (1988) report cellulolytic and methanogenic microbial inhibition in the rumen of foregut-fermenting mammals at a pH of less than 6.0.

The cloacal chamber is a temporary holding site for feces and urine. The cloaca is divided into three regions, 1) coprodaeum, 2) urodaeum, and 3) proctodaeum (Spellerberg, 1982). The coprodaeum is the anterior region of the cloaca and the terminal end of the large intestine, and is the collection site of feces (Porter, 1972). The urodaeum is the middle chamber that connects directly to the bladder and the reproductive tract, and is the collection site of urine (Porter, 1972). The proctodaeum is a short tube that facilitates movement of feces and urine out of the body via a cloacal sphincter to the vent (Porter, 1972; Spellerberg, 1982). The reptilian cloaca also includes a pacemaker that refluxes urine

through the hindgut for host electrolyte and water conservation (Stevens and Hume, 1998), and microbial nitrogen (utilization) conservation. The internal anatomy of the terrestrial tortoise is provided (Fig. 1).

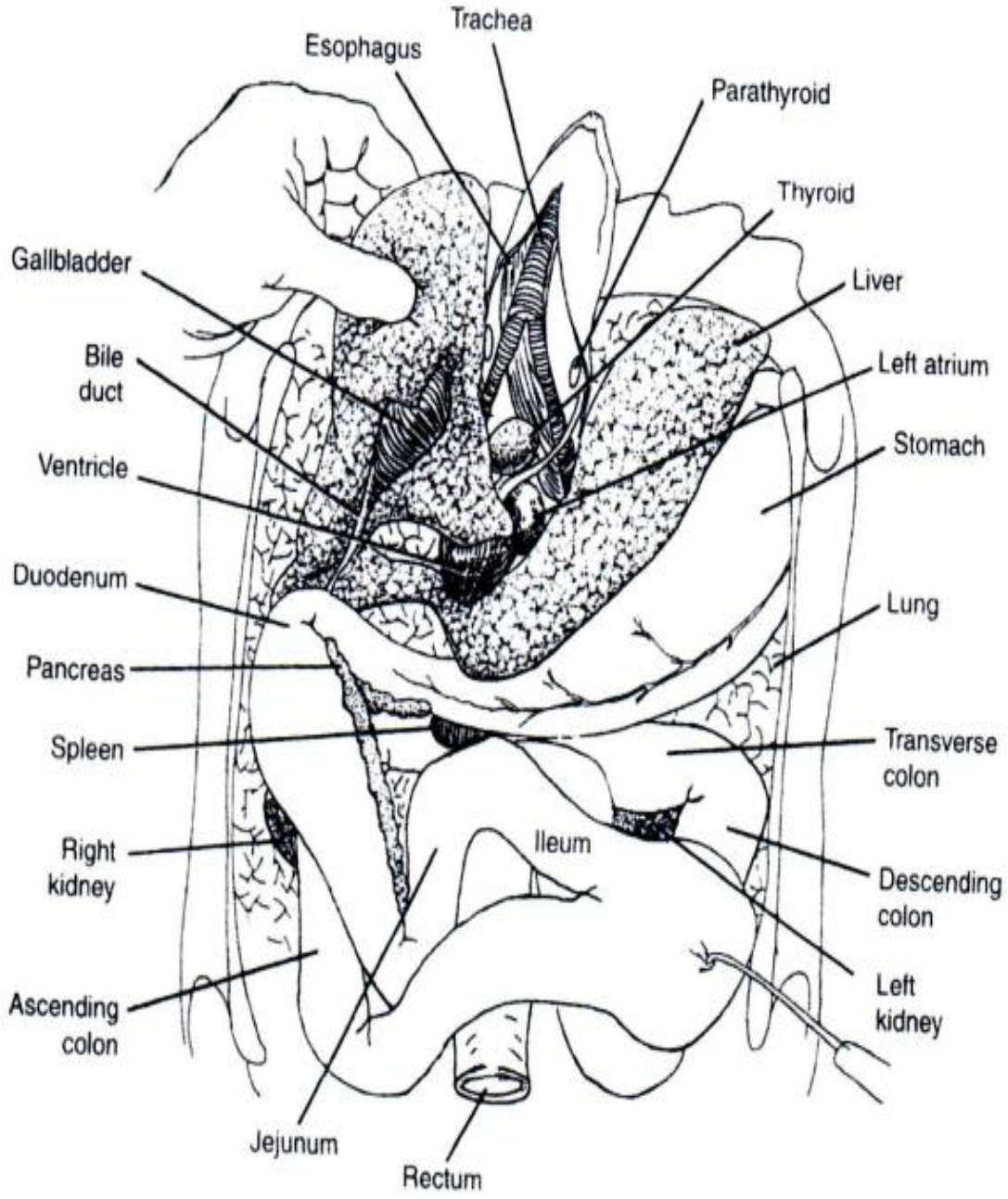

Figure 1. Internal gross anatomy of the terrestrial tortoise (Boyer and Boyer, 2006).

2.3 Hindgut Digestion and Fermentation

Hindgut digestion is similar to chemical digestion occurring in the stomach and small intestine. It is a means of separating insoluble fiber components (i.e., cellulose and hemicellulose) into smaller subunits (i.e., cellobiose and glucose) that are more readily available for uptake by hindgut microbes, resulting in fermentation (Atlas and Bartha, 1998). Cellulose is present in plants as crystalline fibers (Doi and Kosugi, 2004). It is a linear polymer composed of up to 10,000 individual glucose molecules joined together by β-1,4 glycosidic linkages and turned 180°, creating subunits of cellobiose (Schwarz, 2001). Hydrogen bonding between cellulose molecules creates crystallization (Schwarz, 2001). Enzymes secreted directly by the vertebrate gastrointestinal tract are unable to digest the β-1,4 linkages (Van Soest, 1987).

Digestion occurs either by the presence of a cellulosome on the cell surface of anaerobic bacteria and fungi, or by extracellular enzymes secreted by aerobic bacteria and fungi (Bayer et al., 1998, 2008; Schwarz, 2001; Doi and Kosugi, 2004). The cellulosome is a complex of cellulolytic enzymes (β-1,4 endo- and exoglucanases) (Lamed and Bayer, 1986; Andreesen et al., 1989; Bayer et al., 1998; Doi and Kosugi, 2004). The cellulosome has a "string" of glycoproteins that integrate cellulases into the complex (Bayer et al., 1998). The glycoproteins are connected to one another with a dockerin (connecting) domain at one end (Bayer et al., 1998). This domain attaches the glycoprotein "string" to the bacterial cell surface via a cohesion (connecting) domain on an anchoring protein (Bayer et al., 1998). The glycoprotein "string" also hosts a cellulose-specific carbohydrate-binding molecule that attaches to the fiber particle, keeping the fiber and enzymes in close proximity (Bayer et al., 1998). Cellulases disrupt the hydrogen bond between sheets, and

hydrolyze the β-1,4 linkages of the glucose chains, first into cellobiose, then into glucose molecules (Bayer et al., 1998; Schwarz, 2001). The glucose molecules are then transported into the cytosol (although the action of transport has not been defined).

Hindgut fermentation is the process of converting the previously digested nutrients (i.e., glucose) into other substrates (SCFAs). Fermentation is defined as a pathway where a reduced electron acceptor is re-oxidized by metabolites produced in the pathway (White, 1995). The model organism for microbial digestion and fermentation is *Clostridium thermocellum*, and is the basis for the following information (Andreesen et al., 1989; Bayer et al., 1998). Once inside the cytosol, short-chain fatty acids, the end-products of glucose fermentation, will supply energy to the host (Fig. 2).

Glucose first undergoes glycolysis into pyruvate (Andreesen et al., 1989; Van Soest, 1994; Danson et al., 2007). A carboxyl group is removed from pyruvate (decarboxylation), yielding acetyl-CoA, which can travel through two different routes (Andreesen et al., 1989; Van Soest, 1994). First, acetyl-CoA can yield the short-chain fatty acid, acetate, by use of acetyl phosphate (Van Soest, 1994; White, 1995). Acetate is the principal short-chain fatty acid produced from hindgut fermentation of the domestic horse (NRC, 2007). Second, two acetyl-CoA molecules can go through condensation (joining) to yield the short-chain fatty acid, butyrate (Van Soest, 1994; White, 1995). In a separate pathway, pyruvate can, instead, undergo phosphoenolpyruvate fixation of CO_2 (transformation of CO_2 into an organic compound) to yield oxaloacetate (Van Soest, 1994; White, 1995).

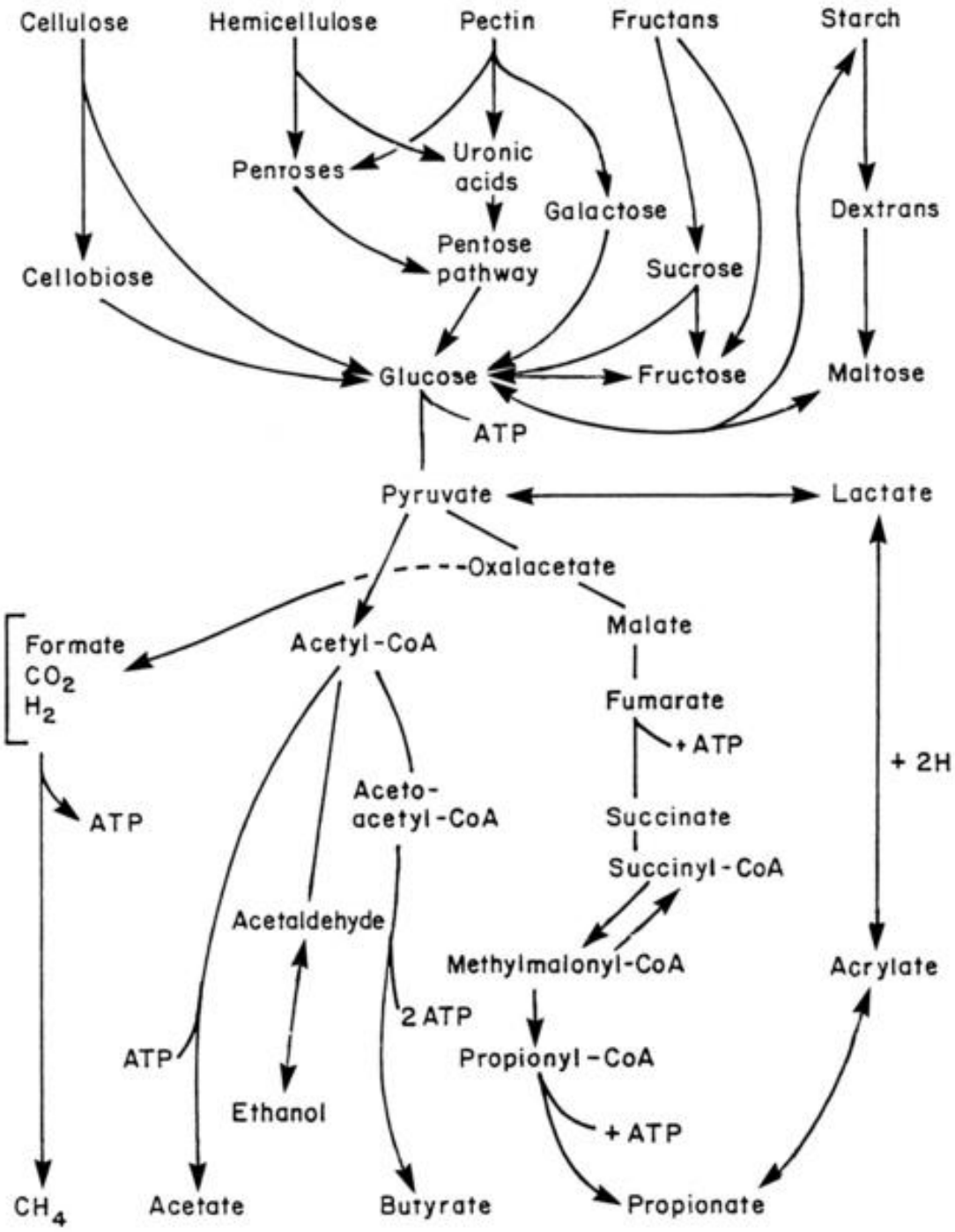

Figure 2. Fermentation pathways of glucose within the bacterial cytosol of the vertebrate (Van Soest, 1994).

Oxaloacetate will feed into a reverse citric acid cycle, beginning with the oxidation of oxaloacetate to malate via the enzyme malate dehydrogenase and the coenzyme NAD^+ (Van Soest, 1994; Salway, 1999). Malate is then converted into fumarate by dehydration (removal of H_2O) with the enzyme fumarase, and fumarate is oxidized into succinate by the enzyme complex succinate dehydrogenase (Van Soest, 1994; White, 1995; Salway, 1999). Succinate is then converted into succinyl-CoA by the enzyme succinyl-CoA synthetase and the addition of a phosphate group (Miller and Wolin, 1979; Van Soest, 1994; White, 1995; Salway, 1999). Succinyl-CoA is isomerized into methylmalonyl-CoA with the enzyme methylmalonyl-CoA mutase and the cofactor vitamin B_{12}; succinyl-CoA and methymalonyl-CoA can convert back and forth to one another (Van Soest, 1994; White, 1995; Salway, 1999). Methylmalonyl-CoA is then carboxylated to the coenzyme ester, propionyl-CoA, via propionyl-CoA carboxylase. The last step is the conversion of propionyl-CoA to propionate via propionyl-CoA transferase, which removes the coenzyme-A (CoA) (Van Soest, 1994; White, 1995; Salway, 1999). This citric acid cycle is "incomplete", as bacterial microbes cannot oxidize acetate into CO_2 and H_2O (Van Soest, 1994). The pathway end-products (SCFAs) are released from the bacterial cytosol, back into the hindgut of the animal. From this point, the SCFAs can be absorbed across the intestinal wall, and supply energy to the animal after aerobic oxidation in the bloodstream (Atlas and Bartha, 1998). Bouchard and Bjorndal (2005) report fermentation capacity (as mass of fermentation contents) of herbivorous reptiles to follow the allometric equation:

$$\text{Capacity, kg} = 0.0926(\text{BW kg})^{0.9919}$$

For example, an herbivorous reptile weighing 10 kg would have fermentation capacity of:

1) $\text{Capacity, kg} = 0.0926(10)^{0.9919}$ 2) $\text{Capacity, kg} = 0.91$

3. METHODOLOGY

The following studies used 16, ten-year-old mature female leopard tortoises (*Stigmochelys pardalis*), born of the same clutch at the Department of Herpetology, Smithsonian National Zoological Park (Washington, DC) in 2005, and received by the Animal Science Department, California Polytechnic State University (San Luis Obispo, CA) in 2008. Use of these animals was reviewed and approved by the California Polytechnic State University Institutional Animal Care and Use Committee (IACUC, Protocol # 903).

Animals were fed one of three 2.5 x 0.9 cm pelleted diets. Researchers were blind to the identity of the two treatment diets; only the control diet was known. The control diet (CNTRL) consisted of Mazuri® Tortoise Diet (5M21, PMI Feeds, St. Louis, MO), formulated for terrestrial herbivorous tortoises. One treatment diet (2.0 mm) consisted of the tortoise diet with the addition of 2.0 mm length cellulose fiber (84.0% and 16.0% by weight, respectively); the second treatment diet (0.2 mm) consisted of the tortoise diet with the addition of 0.2 mm length cellulose fiber (84.0% and 16.0% by weight, respectively). All diets were pelleted at Purina Animal Nutrition (Richmond, IN). All tortoises were fed to satisfy 50% of herbivorous reptile field metabolic rate (FMR, kJ ME/d) based on body weight at the beginning of diet transition (Nagy et al., 1999). Calculated metabolizable energy (ME) from measured gross energy (GE) was 12.49 kJ/g (CNTRL), 12.58 kJ/g (2.0 mm), and 11.99 kJ/g (0.2 mm). Daily food consumption was calculated as the difference between amount offered and orts after 10 h.

Tortoises were transitioned from their previous diet onto Mazuri® Tortoise Diet (5M21) beginning 13 March 2015. From 24 June – 1 July 2015 they were transitioned from

5M21 onto one of the three diets (CNTRL, 2.0 mm, 0.2 mm). Intake at 100% consumption of control or treatment diet occurred at 0900 1 July 2015. There were two phases, 1) an acclimation phase ending d 18, and 2) a collection phase ending d 99.

Using a complete randomized design, diet and fecal particle size distributions as well as change in particle size distribution from diet to fecal state were analyzed, along with apparent digestibility (aDig, %) of selected nutrients, and a novel investigation of the fecal microbiome. Tortoises were randomly assigned to one of the three diets as follows: CNTRL, n = 4; 2.0 mm, n = 6; and 0.2 mm, n = 6. Access to other feed sources and coprophagy was restricted, while drinking water was available *ad libitum*.

Tortoises were housed individually in an open-air building. There were six rectangular enclosures. Four enclosures measured 4.5 x 1.5 m, divided into two pens each. Two enclosures measured 4.5 x 3.1 m, divided into four pens each. Individual pens measured 4.5 x 0.8 m. Enclosures were divided into pens using 39.4 x 24.1 cm cinder blocks or 4.5 x 72.4 cm metal animal fencing with 10.2 cm^2 spacing. For pens with fencing, the first column of the panel was cut down to 43.2 cm tall for easier access between pens. Plastic wire measuring 119.4 x 43.2 cm with 1.3 cm^2 spacing was attached to the metal fencing via cable ties to ensure animals could not reach one another's food dishes through the fencing.

The southern side of the building was covered with 3.6 x 3.0 cm poultry netting 130 cm off the ground and 183 cm tall, offering natural, unfiltered sunlight. Supplemental fluorescent lighting was also provided as a 10L:14D pattern. Each tortoise was provided corrugated plastic pipe shelter measuring 89.5 x 52.5 x 22.5 cm with an entrance on either end. Each tortoise was also provided supplemental heat with a 121.5 x 60.5 cm heat pad

(Stanfield®, Osborne Industries, Inc., Osborne, KS). Heat pads were maintained between 25.0° to 30.0°C during the 0900 h, and 30.0° to 35.0°C during the 1900 h. Minimum, maximum, and current ambient temperatures were recorded twice daily (0900, 1900 h) using a digital thermometer (Acu-Rite®, Chaney Instrument Company, Lake Geneva, WI). Heat pad and concrete floor temperatures were recorded twice daily (0900, 1900 h) using a handheld noncontact infrared thermometer (Raytek Mini Temp MT, Raytek Corporation, Santa Cruz, CA). Average current ambient temperature throughout the trial period was 22.3°C.

Each pen was thoroughly cleaned once per week by scrubbing with antibacterial soap and water. Each pen was also sanitized during the first week of each month by scrubbing with a 30% bleach solution. Daily spot cleaning was performed using a damp cotton towel. Tortoises were removed from their pens during cleaning and temporarily placed, two at a time, in a 50 gal stock tank (Rubbermaid® No. 4243, Newell Rubbermaid Inc., Atlanta, GA).

3.1 Diet Preparation

All diets included the following ingredients, in order, as indicated on the manufacturer information sheets: ground soybean hulls, ground corn, (fiber source), dehulled soybean meal, ground oats, wheat middlings, cane molasses, dehydrated alfalfa meal, wheat germ, dicalcium phosphate, soybean oil, brewers dried yeast, calcium carbonate, salt, dl-methionine, choline chloride, pyridoxine hydrochloride, d-alpha tocopherol acetate, biotin, cholecalciferol, menadione sodium bisulfite complex, calcium pantothenate, vitamin A acetate, folic acid, riboflavin, mixed tocopherols, rosemary extract, nicotinic acid, vitamin B_{12} supplement, thiamine mononitrate, citric acid, l-lysine,

manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, sodium selenite, and cobalt carbonate. Only the 2.0 and 0.2 mm diets included the "fiber source" (powdered cellulose).

Powdered cellulose fiber (Vitacel® Powdered Cellulose, J. Rettenmaier USA LP, Schoolcraft, MI) with average length of 2.0 mm (FIF 400) and 0.2 mm (BWW 40), originating from spruce and pine trees and recommended for use in the pet food industry, was obtained. Cellulose fiber lengths were chosen due to the difference in magnitude, and commercial length availability. Pure cellulose also minimized digestion taking place prior to the hindgut. Approximately 7 kg of each fiber length was run through a dry, vibratory sieve shaker (Retsch® AS 300 control, VERDER Group, Haan, Germany) for particle separation. Cellulose fiber was weighed into plastic weigh boats using a digital balance (A&D FY-3000, A&D Company, Ltd., Tokyo, Japan); 50 g (2.0 mm) and 100 g (0.2 mm) total for each sieving run. Different weights were necessary due to the increased volume associated with increased fiber length. Weighed cellulose fiber was evenly distributed onto the top of a three-sieve stack. Sieve mesh dia were 3.35 mm (305 mm Ø x 40 mm H, Retsch® 60.159.003350), 2.0 mm (305 mm Ø x 40 mm H, Retsch® 60.158.002000), and 1.0 mm (305 mm Ø x 40 mm H, Retsch® 60.158.001000) for the 2.0 mm length fiber; and 0.25 mm (305 mm Ø x 40 mm H, Retsch® 60.158.000250), 0.15 mm (305 mm Ø x 40 mm H, Retsch® 60.158.000150), and 0.125 mm (305 mm Ø x 40 mm H, Retsch® 60.158.000125) for the 0.2 mm length fiber. Once the fiber was transferred to the top sieve, the sieve lid (305 mm Ø x 40 mm H, Retsch 69.520.0051) was placed, followed by the sieve clamping device (Retsch® 32.662.0014).

Sieves were stacked in descending order of mesh size on the sieve collecting pan (305 mm Ø x 40 mm H, Retsch® 69.720.0050). The sieve was run for 5 min at 0.41 mm•"g" amplitude. At the conclusion of the cycle, lids were removed, along with the top sieve, and set aside. The middle sieve (2.0 mm and 0.15 mm) was emptied into an 18 oz sample bag (Whirl-Pak® B01065, Nasco, Fort Atkinson, WI) attached to a 53.3 cm dia plastic funnel. All other sieves were emptied into a waste receptacle. Sample bags were weighed after each sieve run. A new bag was attached to the funnel after collecting 100 to 200 g in a single sample bag. Sieving continued until 1,700 g of each cellulose fiber length was collected.

Collected cellulose was sent to Purina Animal Nutrition (Richmond, IN) for pelleting. Sixteen hundred grams of each cellulose size was added to a separate batch of 8,400 g of Mazuri®'s Tortoise Diet (5M21) to bring the calculated diet NDF concentration to 41.0%. This NDF concentration was chosen as the tortoise colony had previously been fed a low starch tortoise diet (Mazuri® 5E5L) consisting of 41.0% NDF. The 5M21 formula was chosen as the base for the following reasons, 1) the research tortoise colony had been acclimated to it for the past 7 yr, and 2) it does not include any "long-stem" forage that may have competed with the cellulose in the hindgut. Mazuri® tortoise diet (5M21) was ground through a 2.0 mm screen before adding the cellulose and pelleting, to ensure there were no particles larger than 2.0 mm. Diets were received labeled as 5WDJ, 5WDK, and 5WDL. As previously described, only the formula identity of the control diet (CNTRL, 5WDL) was known to the researchers at this time.

Particle separation by size is an approximate analysis since separation through the sieves can be influenced by the rigidity or flexibility of the particles (Fritz et al., 2012).

Therefore, the fiber length referred to as 2.0 mm ranged from 2.0 to 3.35 mm (average of 2.7 mm), and the fiber length referred to as 0.2 mm ranged from 0.15 to 0.25 mm (average of 0.2 mm).

3.2 Diet Transition and Acclimation

Tortoises were transitioned from Mazuri® 5M21 to their assigned diet (CNTRL, 2.0 mm, 0.2 mm) over an 8 d period (24 June – 1 July 2015). Transition occurred as 12.5% per d. Calculated ME (kJ/g) on an as-fed basis (AFB) of 12.70 for 5M21 and 12.80 for CNTRL, 2.0 mm, and 0.2 mm (using calculated ME (kJ/g) of Mazuri® Low Starch Tortoise Diet 5E5L) was used for the first 2 d of transition. Calculated ME (kJ/g, DMB) of 14.19 (CNTRL), 14.29 (2.0 mm), and 13.63 (0.2 mm) were used for d 3 to 4. After the gross energy (GE) content was determined, calculated ME (kJ/g, AFB) of 12.49 (CNTRL), 12.58 (2.0 mm), and 11.99 (0.2 mm) were used thereafter. The first day of 100% test diet ingestion occurred on 1 July 2015. On 2 July 2015, approximately 24 h after ingestion of 100% test diet, pellets of each tortoise's diet were prepared with 100 indigestible, 2.0 mm dia acetate beads (Engineering Laboratories, Inc., Oakland, NJ) by moistening the pellet, kneading in the beads, and re-forming the pellet. At 0925 h, each tortoise was individually hand-fed the pellets containing the beads. Excretion of markers in the feces was used as an indication the ingested test diet was represented in those feces (Hamilton and Coe, 1982). Range of first marker appearance was 7 to 41 d.

Multiple measures were put in place to minimize the risk of feeding the diets incorrectly. Each diet was assigned a color: CNTRL was assigned white, 2.0 mm was assigned red, and 0.2 mm was assigned green. Diets were placed into their own color-coordinated bin with a color-coordinated Velcro® wrap used to keep the plastic diet bags

sealed when not in use. Each individual tortoise pen had an 8.5 x 11" piece of colored paper, corresponding to their assigned diet, laminated and hung at the end of their pens to easily indicate diet type per individual animal. White food bowls were marked on the back with an "x" in color so that the same set of bowls was used for each diet type throughout the trial (6 "red" bowls, 6 "green" bowls, and 4 "white" bowls). Diet colors were also written above their respective codes on the daily feed sheets. Diet was weighed, stored, and fed out one diet type at a time. For example, CNTRL would be 1) weighed out for the 4 animals on that diet, 2) the diet bag would be sealed and put away in its bin, and 3) those food bowls would be given to each of the corresponding animals before another diet type was weighed and fed.

3.3 Total Fecal Collection Harness Application

Each tortoise was individually fitted with a total fecal collection harness which allowed for collection of uncontaminated fecal samples. Harnesses were applied on 17 June 2015 to terminate any possible recycling of feces via coprophagy. The nylon harnesses measured 17.5 cm long, 11.5 cm wide at the base, and 15.5 cm wide at the top opening that attached to the tortoise. There were 36 harnesses available; 18 of each color (black and blue). Harnesses were numbered at the top from 01 to 18. To aid in control versus treatment diet recognition, tortoises assigned to the CNTRL diet were assigned black harnesses, and tortoises assigned to the 2.0 and 0.2 mm diets were assigned blue harnesses. Harnesses were attached to the caudal end of the carapace and plastron via industrial strength hook and loop fasteners (Velcro® 847062, Pleasant Prairie, WI). Before fastener application, all tortoises were scrubbed with water and a soft brush along the caudal portions of the shell to ensure both the carapace and plastron were clean. Two fastener pieces measuring

approximately 2.56 x 2.22 cm were attached to left and right sides of the supracaudal scute, midway between the cranial and caudal ends of the scute. One fastener piece measuring approximately 9.95 x 2.50 cm was attached over the caudal end of the femoral scutes and cranial end of the anal scutes. Harness identification numbers and weights were recorded.

3.4 Sample Collection

Beginning on 2 July 2015, fecal collection occurred at 0900, 1400, and 1900 h for 99 d. Initial defecation of feces containing indigestible markers was collected and measured but not used for analysis. Subsequent samples were collected until a minimum 100 g was collected from each animal. Harness weights (with sample and after sample removal), harness identification numbers, fecal sample weights (by difference), and collection date and times were recorded. Urine was also recorded. Full (with fecal sample) harnesses were removed from the tortoises. Residual feces on the tortoises' body was removed and added to the harness. Full harnesses were weighed. Feces were removed from the harnesses as thoroughly as possible, and transferred to labeled re-closable bags. Empty, soiled harnesses were also weighed. Sample amount was calculated as the difference between the full harness weight and harness tare weight. Sample bags were stored in a refrigerator on-site, and transferred, within 24 h of collection, to a freezer (-20.0°C) in the Cal Poly Animal Nutrition laboratory until analysis. Clean harnesses were weighed and attached to the tortoises. Soiled harnesses were hand washed using water and a scrub brush, and air-dried.

4. PHYSICALLY EFFECTIVE FIBER

While the term “dietary fiber” is commonly used in human nutrition, it does not discriminate against the different types of carbohydrates (nonstructural and structural) that make up “fiber”. Terms more commonly used in animal nutrition include “soluble fiber” and “insoluble fiber”, each of which represents specific parts of the plant cell wall and the role they fulfill in the gastrointestinal tract. Soluble fiber (nonstructural carbohydrates) includes starch, pectins, gums, and β-glucans that serve as bonding agents in the cell wall (Van Soest, 1987, 1994). These components can be digested, to a large extent, by the host’s own enzymes due to the presence of α-1,4 glycosidic linkages between glucose monosaccharides (Van Soest, 1994). Insoluble fiber (structural carbohydrates) includes cellulose, hemicellulose, and lignin (collectively referred to as neutral detergent fiber, NDF) that provide the cell with structure, and the plant with stability. These components are indigestible by the host’s own enzymes due to β-1,4 glycosidic linkages (Van Soest, 1994). Two components of NDF, cellulose and hemicellulose, can only be digested by enzymes produced by symbiotic microorganisms capable of cleaving β-bonds that inhabit the host’s gastrointestinal tract; lignin is indigestible (Van Soest, 1994).

Neutral detergent fiber (NDF) components play two roles within the gastrointestinal tract, regarded as ‘nutritionally effective’ and ‘physically effective’ (Van Soest, 1994). Neutral detergent fiber that fulfills a nutritionally effective role provides the host with energy in the form of short-chain fatty acids (Van Soest, 1994; Atlas and Bartha, 1998; Donoghue and McKeown, 1999; Stevens and Hume, 2004) and nutrients in the form of B vitamins and vitamin K (Atlas and Bartha, 1998; Hooper et al, 2002), and essential amino acids (Atlas and Bartha, 1998; Arthur et al., 2014) from microbial digestion and

fermentation. Neutral detergent fiber (NDF) that fulfills a physically effective role adds bulk to the diet, resulting in a slower rate of digesta passage and allowing more time for microbial digestion and fermentation to take place (NRC, 2007). Neutral detergent fiber should be provided in the diet in sufficient amounts such that both roles are fulfilled simultaneously. However, these amounts have yet to be objectively quantified.

Developed for ruminants, using dairy cattle as a model species, physically effective neutral detergent fiber (peNDF) is defined as a measurement of dietary fiber based on both NDF concentration and particle length of a given feed substrate (Mertens, 1997). The purpose of peNDF in the ruminant is to regulate rumination by increasing chewing activity, subsequently increasing salivary buffer secretion, and maintaining ruminal pH and an adequate microbial environment (Mertens, 1997). For dairy cattle, Mertens (1997) recommends ≥ 25% NDF on a dry matter basis (DMB), of which ≥ 19% should be peNDF of ≥ 1.18 mm length particles. For adult, hindgut-fermenting reptiles, Donoghue and McKeown (1999) recommend 18 to 28% crude fiber (CF, DMB); however, fiber particle length (peNDF) is not considered in this recommendation.

The purpose of peNDF in the non-ruminant has yet to be defined (NRC, 2007), as it has been previously suggested that digestive processes alone have little influence on particle size reduction (Poppi et al., 1981). However, more recent studies appear to refute this. Fritz et al. (2010) implicated increased retention time of reptilian hindgut-fermenters as a means for gastrointestinal tract particle size reduction. It has also been suggested that particle size is important for hindgut homeostasis, as homeostasis can be compromised when increased amounts of "small" fiber particles are introduced to the hindgut due to their high digestibility and rapid fermentation rates (NRC, 2007), although "small" has yet to be

defined. Therefore, peNDF may be necessary for non-ruminant herbivores as a means of slowing digesta movement in the hindgut, allowing more time for microbial fermentation and maintaining a healthy hindgut environment. We have developed a working definition of peNDF for non-ruminant hindgut-fermenting vertebrates as follows: physically effective neutral detergent fiber is the combined influence of fiber particle length and NDF concentration that promotes hindgut motility as it relates to microbial fermentation of structural carbohydrates.

Measuring and managing diet particle length is a way to ensure peNDF is being provided sufficiently, but there must also be a way to measure its impact in the gastrointestinal tract. Measuring fecal particle size may be an indication of these influences. Fecal particle size has been used, primarily, as a determinant of the chewing efficiency of ruminants (Fritz et al., 2009), and is often reported in terms of mean fecal particle size (MPS) (Hummel et al., 2008; Fritz et al., 2009, 2010; Clauss et al., 2009, 2015), calculated by weighted average of recovered particles or by use of a fitted curve to the particle size distribution (Fritz et al., 2010). However, in non-ruminant hindgut-fermenters, mastication complicates the use of fecal particle size as a measurement of peNDF efficiency, since peNDF in the hindgut-fermenter is concerned with fiber particle length and action in the latter portion of the gastrointestinal tract.

Therefore, fecal particle size as a measurement of peNDF influence may be most applicable when diet is also profiled for particle size distribution, and ingesta versus excreta particle size can be compared. Published data on total diet and fecal particle size distribution is lacking. As of this review, the author is aware of two studies involving non-ruminant ingesta and fecal/digesta particle size comparisons, 1) average diet and fecal

particle size (MPS) of 17 domestic horses fed one of three hay diets (Carmalt and Allen, 2008), and 2) diet and duodenal digesta particle size proportions (% dry weight) of 240 male broiler chicks fed a mashed or pelleted wheat-based diet (Amerah et al., 2007).

In a comparison of reptile fecal particle size with fecal particle size of captive herbivorous non-ruminant mammals from a previous study, Fritz et al. (2010) concluded that reptiles produce larger fecal particles than mammals, with fecal particle size increasing with increasing body mass in both clades. Furthermore, reptile fecal particle size was even greater than mammals of greater body mass (Fritz et al., 2010). This may be due to the mechanical particle size reduction capability of mammals (mastication) that reptiles lack, leading to larger ingesta particle size of reptiles. Reptile particle size reduction is therefore associated with increased digesta retention time and the digestive role of hindgut microbes.

A compilation of herbivorous hindgut-fermenting reptile (Table 1) and mammal (Table 2) fecal particle sizes are reported. Reptile fecal particle sizes are larger than mammals on comparable diets of hay and other items, but smaller in reptiles fed an extruded commercial diet only.

Table 1. Mean fecal particle size and diet of captive and free-ranging herbivorous, hindgut-fermenting reptiles.

Species	Sample Size (*n*)	Diet	Mean Fecal Particle Size (mm)	Source
Corucia zebrata	10	Captive diet[1]	17.66[a]	Fritz et al., 2010
Dipsochelys dussumieri	6	Captive diet[1]	32.19[a]	Fritz et al., 2010
Geochelone nigra	7	Captive diet[1]	26.80[a]	Fritz et al., 2010
Testudo gigantean	5	Captive diet[1]	59.74[a]	Fritz et al., 2010
T. radiata	3	Captive diet[1]	14.51[a]	Fritz et al., 2010
T. sulcata	3	Captive diet[1]	19.21[a]	Fritz et al., 2010
T. horsefieldii	1	Captive diet[1]	9.25[a]	Fritz et al., 2010
T. hermanni	10	Chopped parsley and grass hay[2]	2.74[a]	Fritz et al., 2010
	10	Whole parsley and grass hay[2]	6.71[a]	Fritz et al., 2010
	7	Natural diet[3]	3.12[a]	Fritz et al., 2010
T. graeca	9	Chopped parsley and grass hay[2]	4.49[a]	Fritz et al., 2010
	9	Whole parsley and grass hay[2]	9.16[a]	Fritz et al., 2010
	5	Natural diet[3]	2.28[a]	Fritz et al., 2010
Stigmochelys pardalis	4	Captive diet[1]	24.18[a]	Fritz et al., 2010
	6	Extruded feed (Mazuri® 5M21)[2]	0.27[b]	Author's unpublished data
	6	Extruded feed (Mazuri® 5E5L)[2]	0.76[b]	Author's unpublished data

[1]Captive individuals fed varying mixtures of browse, "hays", herbs, leaves, "salad", fruits, and vegetables
[2]Captive individuals
[3]Free-ranging individuals
[a]Particle size reported as mean particle size (MPS)
[b]Particle size reported as discrete mean (dMEAN)

Table 2. Mean fecal particle size and diet of captive herbivorous, hindgut-fermenting mammals.

Species	Sample Size (*n*)	Diet	Mean Fecal Particle Size (mm)	Source
Equus caballus	8	Grass hay and whole oats	1.09[a]	Carmalt et al., 2005
	8	Grass hay and oat hull pellets	0.95[a]	Carmalt et al., 2005
	8	Grass hay and fat pellets	0.90[a]	Carmalt et al., 2005
	8	Grass straw and whole oats	0.96[a]	Carmalt et al., 2005
	8	Grass straw and oat hull pellets	0.90[a]	Carmalt et al., 2005
	9	Timothy hay and oats	0.39[a]	Carmalt and Allen, 2006
	9	Timothy hay and soy pellets	0.36[a]	Carmalt and Allen, 2006
	8	Timothy hay, canola meal pellets	0.37[a]	Carmalt and Allen, 2006
	8	Timothy hay	0.39[a]	Carmalt and Allen, 2006
	17	"Hay" diet (3 types)	1.36[a]	Carmalt and Allen, 2008
	5	Grass hay	1.12[b]	Clauss et al., 2015
	3	Grass hay	1.07[b]	Clauss et al., 2015
E. greyvi	4	Grass hay	1.55[b]	Clauss et al., 2015
E. przewalskii	3	Grass hay	1.20[b]	Clauss et al., 2015
Loxodonta africana	5	Grass hay	4.98[b]	Clauss et al., 2015
Phacochoerus africanus	1	Grass hay	1.22[b]	Clauss et al., 2015
Ceratotherium simum	1	Grass hay	5.10[b]	Clauss et al., 2015

[a]Particle size reported as mean particle size (MPS)
[b]Particle size reported as discrete mean (dMEAN)

The objective of this study was to investigate particle size reduction of two known cellulose fiber particle lengths (2.0 and 0.2 mm) in the hindgut of *S. pardalis* using diet and fecal particle size distributions as measures for analysis of physical effectiveness. We hypothesized, based on our definition of peNDF for hindgut-fermenting vertebrates, that peNDF would not be digested in the hindgut to a significant extent, therefore appearing in the fecal particle size distributions with a significant difference in recovered particles at 2.0 and 0.125 mm sieves, based on diet. Results may be applicable to the animal feed industry where finely ground ingredients are often used in pelleted and extruded diets designed for herbivorous hindgut-fermenting vertebrates.

4.1 Materials and Methods

4.1.1 Particle Size Determination

Diet - All diets (CNTRL, 2.0 mm, 0.2 mm) were analyzed for particle size distribution. Samples were collected from original batches on 24 Jun 2015. Two 30 g (30.0000 to 30.9999 g) subsamples were analyzed for particle size. Run order of diet type was randomized. Each subsample was placed in a 1,000 mL beaker with 600 mL of deionized (DI) water. The beaker, covered with plastic food wrap to avoid contamination, was soaked for 24 h (Clauss et al., 2002; Hummel et al., 2008; Fritz et al., 2010a, 2012) under refrigeration (5°C). Refrigeration was chosen to retard microbial growth in the solution. After 24 h, an octagonal stir bar was carefully added, and contents were stirred, uncovered, at 700 RPM on a stir plate (Corning® PC-620D, Corning Inc., Corning, NY) for 1 h to homogenize the solution.

The homogenized solution was poured evenly onto the top of a six sieve stack on a Retsch® AS 300 Control vibratory sieve shaker. Sieve mesh diameters were 2.0 mm

(Retsch® 60.158.002000), 1.0 mm (Retsch® 60.158.001000), 0.5 mm (Retsch® 60.159.000500), 0.25 mm (Retsch® 60.158.000250), 0.125 mm (Retsch® 60.158.000125), and 0.063 mm (Retsch® 60.159.000063). Also included in the sieve stack were a collecting pan with outlet (Retsch® 69.430.0050), a ventilation ring (Retsch® 69.321.0050), and an intermediate ring (Retsch® 60.935.000305). Sieves were stacked in descending order of mesh size with the intermediate ring above the 2.0 mm sieve, the ventilation ring between the 0.25 and 0.125 mm sieves, and the collecting pan below the 0.063 mm sieve. After pouring the sample, the clamping device (Retsch® 32.662.0014) was placed onto the sieve stack. The sieve shaker was run for 5 min at 0.41mm•"g" amplitude, running 2 L•min^{1} filtered tap water through the sieve stack. Water flow rate was calibrated using a Gems™ rate meter (Gems™ M103 Series Compact Rate Meter/Totalizer, Gems Sensors Inc., Plainville, CT). Material that passed through the smallest sieve size (0.063 mm) exited a hose attached to the collecting pan under the sieve, and was collected into a 4,000 mL plastic Nalgene® beaker.

After the run time, each sieve (and corresponding ring) was individually rinsed of any remaining material into 10 x 20 cm, 0.05 mm porosity, pre-weighed forage bags (Ankom®, R1020, Macedon, NY). Forage bags were attached via Velcro® to the neck of a 53.3 cm dia funnel. Sieves and rings were rinsed thoroughly with tap water into the funnel. The material that escaped the 0.063 mm sieve was also poured into a pre-weighed forage bag where any material > 0.05 mm was recovered. After each sieve and ring was rinsed, they were individually placed into an ultrasonic bath (Retsch®, U2, VERDER Group, Haan, Germany) filled with DI water for 1 min to remove residue.

Forage bags with recovered particulate were placed into a 100°C forced air drying oven (Blue M ESP-400BC-4, Blue M Electric Company, Blue Island, IL) for 24 h. After 24

h, forage bags were removed, allowed to cool in a desiccator for 1 h, and weighed (Mettler® XS205, Mettler-Toledo International, Inc., Columbus, OH). Dry sample weight was calculated as the forage bag and dry sample weight minus the dry, empty forage bag weight.

Feces - Run order by tortoise ID was randomized. Frozen fecal samples were pooled into a single composite sample for each individual tortoise. Each sample bag was peeled down around the frozen fecal sample until the fecal sample could be removed and placed into a labeled gallon size re-closable bag. Pooled fecal samples were thawed for 72 h under refrigeration before analysis. All fecal particle size analyses were run in triplicate. Once thawed, samples were homogenized by physical kneading of the sample in the gallon bag, and three 30 g (30.0000 to 30.9999 g) subsamples were removed. One subsample was processed per day. All subsamples from a single tortoise were run over three consecutive days. Subsequent steps were followed as described for "diet" particle size analysis described above.

4.1.2 Statistical Analysis

Coefficient of variation (CV) with an acceptable limit ≤ 0.05 was used as a reliability threshold of particle size distribution results among duplicate samples. Multivariate analysis of variance (MANOVA) using the Wilks-λ statistic in JMP Pro 12 (SAS Institute Inc., Cary, NC) was used to compare particle size distributions (fecal and change from diet to fecal state) between diet. Differences were considered statistically significant at $P < 0.05$. If the model was statistically significant, Tukey's pairwise comparison was used to compare the particle size data by sieve size.

The discrete mean particle size (dMEAN), a form of a weighted average reported in mm, was calculated for diet and feces using the method described by Fritz et al. (2012).

This method has been employed in other fecal particle size studies since its publication (Clauss et al., 2015; Matsuda et al., 2014). Sieves are ordered by pore size (S), from smallest ($S(1)$) to largest ($S(n)$). The proportion of particles retained on a given sieve ($p(i)$) includes those particles smaller than $S(i + 1)$, but not larger. A dMEAN value is calculated for each sieve size and multiplied by its respective sieve proportion. The resulting values are added together to calculate a single dMEAN for a given sample.

$$dMEAN = \sum_{i=1}^{n} p(i) * \frac{S(i+1) + S(i)}{2}$$

4.2 Results

4.2.1 Particle Size Distribution

Diet - Mean dry matter distribution (%DM) of particles was determined for each diet (Fig. 3). There was greater recovery, numerically, of particles on the 2.0 mm sieve for the 2.0 mm diet than other diets. Similarly, there was greater recovery, numerically, of particles on the 0.125 mm sieve for the 0.2 mm diet than the others. The CNTRL diet retained the greatest number of particles on the 0.5 mm sieve. This amount was greater than the amount recovered for either cellulose-added diet on the same sieve. The dMEAN calculated for each diet was: 0.50 mm (2.0 mm), 0.45 mm (CNTRL), and 0.43 mm (0.2 mm).

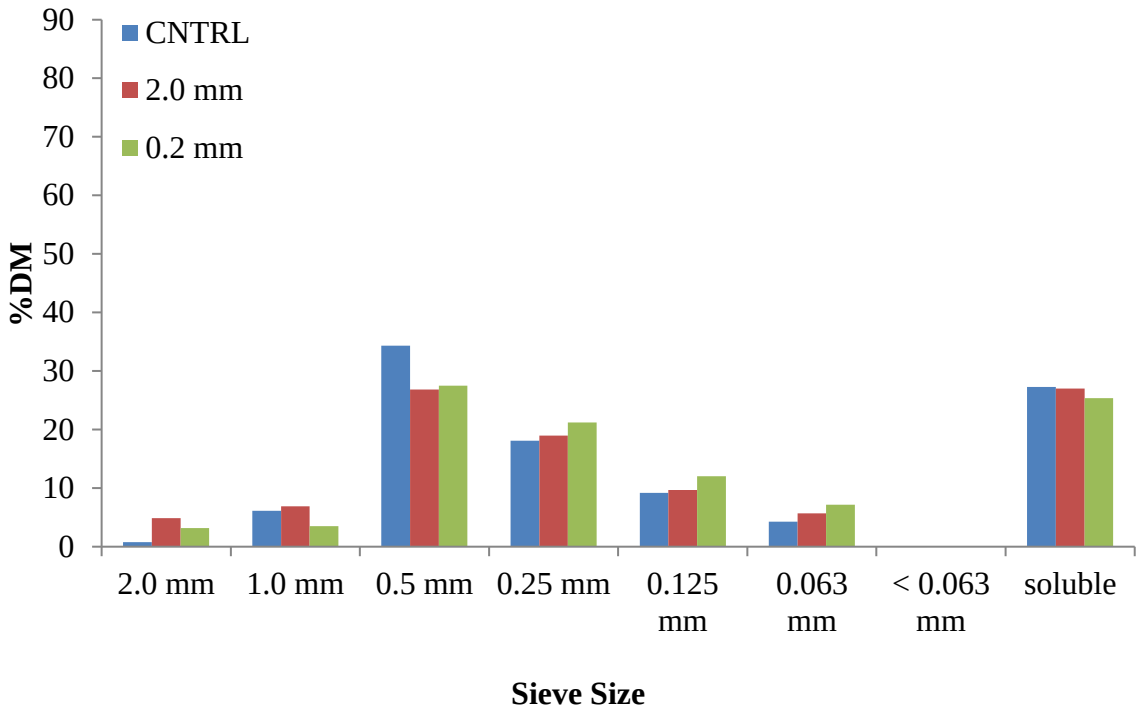

Figure 3. Mean dry matter distribution (%) of a pelleted herbivorous tortoise diet (CNTRL), and the same diet with added 2.0 or 0.2 mm length cellulose fiber.

Feces - Mean (± standard error of the mean, SEM) dry matter distribution (%DM) of fecal particles was determined for each diet (Fig. 4). The MANOVA model revealed no significant difference ($P = 0.1227$) between the three average fecal distributions (CNTRL, 2.0 mm, 0.2 mm). The dMEAN was calculated for each (average) fecal distribution by diet. The CNTRL diet exhibited the greatest dMEAN, and the 0.2 mm diet exhibited the smallest dMEAN. In descending order, the dMEAN (± standard deviation, SD) are: 0.18 ± 0.015 mm (CNTRL), 0.16 ± 0.012 mm (2.0 mm), and 0.15 ± 0.011 mm (0.2 mm); values are significantly different by diet ($P < 0.01$).

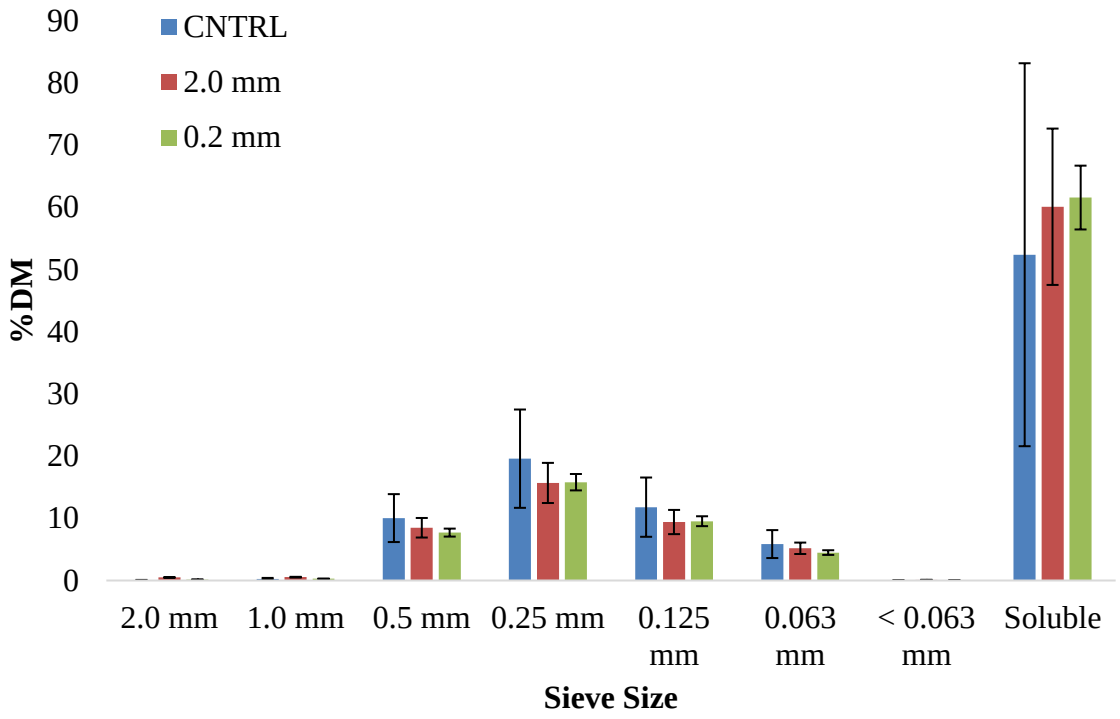

Figure 4. Mean dry matter distribution (%) (± SEM) from feces of leopard tortoises fed a nutritionally complete, pelleted herbivorous tortoise diet (CNTRL, n = 4), and the same diet with added 2.0 mm (n = 6) or 0.2 mm (n = 6) length cellulose fiber.

Change in Particle Size - Mean (±SEM) dry matter distribution (%DM) of particle size change from diet to fecal state was determined for each diet (Fig. 5). The MANOVA revealed a significant difference ($P < 0.05$) among the three change distributions. Therefore, differences in the distributions at each individual sieve size were investigated by use of Tukey's pairwise comparison. Only one sieve (< 0.063 mm) revealed no significant difference ($P = 0.2198$) among the three diets. The dMEAN was calculated for each change distribution by diet. The 2.0 mm diet exhibited the greatest change in dMEAN, and the CNTRL diet exhibited the smallest change in dMEAN. In descending order, the dMEAN (± SD) are: 0.33 ± 0.012 mm (2.0 mm), 0.29 ± 0.011 mm (0.2 mm), and 0.27 ± 0.015 mm (CNTRL); values are significantly different by diet ($P < 0.01$).

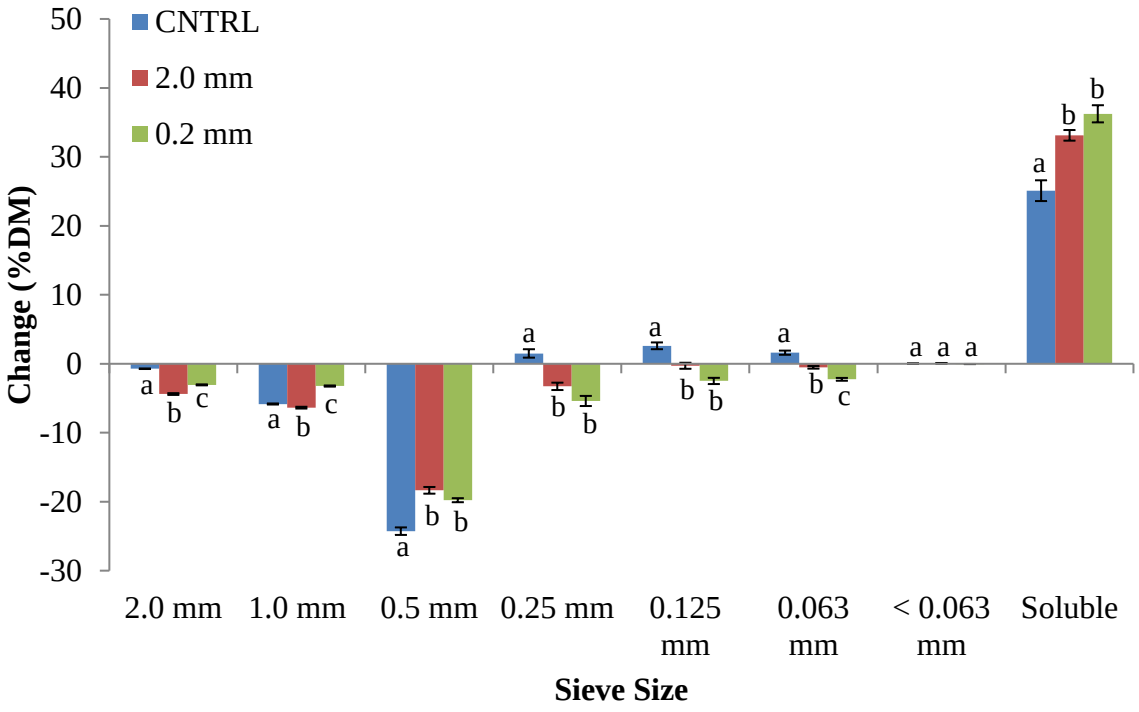

Figure 5. Mean distribution of change (% DM, ± SEM) in particle size of a nutritionally complete, pelleted herbivorous tortoise diet (CNTRL, $n = 4$), and the same diet with added 2.0 mm ($n = 6$) or 0.2 mm ($n = 6$) length cellulose fiber to feces of leopard tortoises fed one diet exclusively. Different letters indicate significant differences ($P < 0.05$).

4.3 Discussion

4.3.1 Particle Size Distribution

Diet - Particle size distribution trends and dMEAN for each diet were as expected; the greatest amount of recovered 2.0 mm particles correlated with the 2.0 mm diet, and the greatest amount of recovered 0.125 mm particles correlated with the 0.2 mm diet. This demonstrates reliability of the wet sieving method to recover particles of known length from a source of unknown particle size distribution.

However, we were unable to test for significant differences among the diet distributions since there was only one batch (observation) for each diet. The ability to test for significant differences among the diet distributions may be beneficial in future studies, as it is possible that although the diet profile trends exhibited what was expected at the 2.0

mm and 0.125 mm sieves, the amounts may not have been significantly different from one another. This could impact the interpretation of the resulting fecal particle size distributions.

Diet particle size has not traditionally been included in fecal particle size studies. Diet particle size analysis could be problematic for studies utilizing free-ranging animals (Hummel et al., 2008; Millette et al., 2012; Matsuda et al., 2014), where sample collection of selected material would not be representative of the ingested material size of the study species. However, diet particle size may be an important feature for future studies to consider including, as it allows the researcher to determine particle size change along the gastrointestinal tract. Although it may be assumed that some degree of particle size change occurs between ingestion and excretion, empirical data of change serves as actual evidence of the particle size-reducing capability of the gastrointestinal tract. This data can help researchers better understand how diets are processed by the gastrointestinal tract, and how that may translate to captive feeding protocols and overall captive animal husbandry and management.

Feces and Change - Fecal particle size distributions were different than expected. We hypothesized that if one of the cellulose particle sizes acted as peNDF, it would be represented in the fecal particle size distribution with significant differences occurring at one or both of the 2.0 mm and 0.125 mm sieves. However, no significant difference among the three fecal particle size distributions were detected.

Numerical changes in particle size distribution from diet to fecal state by diet were different than expected. The greatest reduction of particle size from diet to fecal state at the 2.0 mm sieve size occurred with the 2.0 mm diet. Additionally, the greatest reduction of

particle size from diet to fecal state at the 0.125 mm sieve size occurred with the 0.2 mm diet. Based on our hypothesis, if either the 2.0 mm or 0.2 mm length cellulose acted as peNDF, then the 2.0 mm and 0.2 mm diets should have exhibited the least reduction of particle size on the 2.0 mm and 0.125 mm sieve sizes, respectively. The dMEAN values were also not as expected. Based on our hypothesis, if either the 2.0 mm or 0.2 mm length cellulose acted as peNDF, we would expect the least amount of change in particle size from diet to fecal state for either the 2.0 mm or 0.2 mm diet, respectively.

Fecal distribution similarity suggests both cellulose lengths are below the peNDF threshold for leopard tortoises. This also suggests the peNDF threshold for leopard tortoises is greater than the threshold for mammalian, ruminant, foregut-fermenters (≥ 1.18 mm; Mertens, 1997). A greater peNDF threshold for hindgut-fermenting vertebrates compared to ruminant, foregut-fermenting vertebrates is conceivable, as hindgut-fermenting vertebrates excrete significantly greater-sized fecal particles than foregut-fermenting vertebrates, and lack the ability to ruminate. Additionally, particle size reduction along the gastrointestinal tract of the herbivorous, hindgut-fermenting green iguana (*Iguana iguana*) has been demonstrated (Fritz et al., 2010).

Fed grass hay *ad libitum*, Clauss et al. (2015) demonstrated greater average fecal particle size of 7 hindgut-fermenting mammal species (dMEAN = 2.32 mm) compared to 9 ruminant, foregut-fermenting mammal species (dMEAN = 0.39 mm). This difference is most likely attributable to the greater degree of mechanical particle size processing achieved by ruminant animals, and by the limitation of the reticulo-omasal orifice diameter, through which all digesta particles must pass. The physiological difference in the order of

how food is processed between foregut-fermenting and hindgut-fermenting vertebrates should be further explored with regards to peNDF.

The basis of peNDF for ruminant, foregut-fermenters is to provide fiber of great enough length to stimulate rumination (regurgitation and mastication), and secrete salivary buffers that aid in rumen pH maintenance for the health of rumen microbes (Mertens, 1997), with the understanding that these "large" particles will eventually be processed into smaller particles (by the combination of rumination and microbial digestion). Particles ≥ 1.18 mm were chosen as the peNDF threshold for ruminants, because they are too large to pass through the reticulo-omasal orifice (Mertens, 1997), and therefore, require additional processing (rumination).

Rumination serves two purposes: it stimulates salivary buffer secretion while simultaneously reducing particle size. This manages the rumen pH due to the continual production of acids during fermentation, while also creating fiber lengths (surface area) that are more easily attacked by rumen microbes (Owens and Goetsch, 1988; Bjorndal et al., 1990). It is an efficient means of particle size reduction, because only coarse particles are specifically selected for rumination (Owens and Goetsch, 1988). Therefore, particles leaving the rumen and traveling through the rest of the gastrointestinal tract to excretion should be < 1.18 mm, explaining the recovery of small fecal particle sizes. Comparing hindgut-fermenter fecal particle size to ruminant, foregut-fermenter fecal particle size may not be the most accurate representation of gastrointestinal tract particle-size reducing capability, as it should always be expected that ruminant fecal particle size is < 1.18 mm (Table 3).

Table 3. Body mass (kg) and fecal particle size (dMEAN) of captive ruminant, foregut-fermenting (FF) mammals and hindgut-fermenting (HF) mammals fed grass hay *ad libitum* (Clauss et al., 2015).

Digestive Strategy	Species (*n*)	Total Individuals (*n*)	Body Mass Range (kg)	dMEAN Range (mm)
FF	9	22	60 - 1287	0.26 – 0.54
HF	7	22	77 - 4000	1.07 – 5.10

Due to the lack of rumination, it can be expected that hindgut-fermenting vertebrates have larger ingesta particles than ruminant, foregut-fermenting vertebrates. It can also be expected that herbivorous tortoises have larger ingesta particles than herbivorous mammals, since tortoises completely lack the particle size-reducing action of mastication (Norman and Weishampel, 1985; Fritz et al., 2010). Although teeth are present in the herbivorous *I. iguana*, and some amount of mechanical particle size reduction most likely occurred, substantial particle size reduction was demonstrated in the gastrointestinal tract with greater particle size in the stomach (45 mm) versus the hindgut (5 mm) (n = 1, Fritz et al., 2010) – a nine-fold reduction in particle size. Furthermore, all sections of the *I. iguana* hindgut revealed similar particle sizes (Fritz et al., 2010), suggesting there may also be a minimum fiber length threshold for microbial digestion and fermentation. Substantial particle size reduction in the hindgut may best be explained by selective retention of larger particles and subsequent microbial action, allowing reduction of larger particles (Bjorndal et al., 1990; Barboza, 1995).

Substantial particle size reduction from diet to feces has also been demonstrated in 17 adult domestic horses (*Equus caballus*) (Table 4). In comparison with *I. iguana*, a greater amount of particle size reduction may be attributable to mastication, as there was no significant difference between mean stomach and mean fecal particle size, while a substantial difference between mean diet particle size and stomach/fecal particle sizes was

evident (Carmalt and Allen, 2008). Nutrient concentrations were not considered different from one another based on diet, but mean particle size of each diet was significantly different from the others (Carmalt and Allen, 2008). Fecal particle sizes were not significantly different based on diet (mean of 1.36 mm, with a range across all diets of 1.15 to 1.72 mm) (Carmalt and Allen, 2008), although they were much smaller than their respective mean diet particle sizes. The extent of the influence of mastication on particle size may be a significant difference between herbivorous reptiles and mammals. The significant difference between mean diet particle sizes coupled with similar mean fecal particle sizes further corroborates the idea of a minimum fiber length threshold in addition to a maximum.

Table 4. Fiber fractions (NDF, ADF) and mean diet particle size of three hay diets fed to 17 adult domestic horses (*Equus caballus*) (Carmalt and Allen, 2008).

Diet	%NDF[1], DMB	% ADF[1], DMB	Mean diet particle size (mm)[2]
1	62.8	53.3	43.09[a]
2	55.5	48.1	50.62[b]
3	53.8	44.1	74.40[c]

[1]NDF, neutral detergent fiber (cellulose, hemicellulose, lignin); ADF, acid detergent fiber (cellulose, lignin)
[2]Different letters indicate significant differences at $P < 0.001$

5. APPARENT DIGESTIBILITY

The term "digestibility" is defined in animal nutrition as the percentage of a feed (or any single nutrient) acted on in the digestive tract to a point that it can be absorbed and used by the body's cells (Schneider and Flatt, 1975). Digestibility can be divided into "true" and "apparent". True digestibility takes into account endogenous losses (i.e., sloughed intestinal cells, microbial material) and measures the percentage of a feed (or nutrient) that is absorbed by the body (Schneider and Flatt, 1975). In contrast, apparent digestibility does not take into account the percentage of feed (or nutrient) absorbed, but rather the percentage of feed (or nutrient) ingested and excreted (Schneider and Flatt, 1975). Apparent digestibility is therefore an approximation compared to true digestibility.

In vivo digestibility studies are imperative to understanding the total value of a given feed (Schneider and Flatt, 1975), since feed components are not utilized equally by the body. Traditionally, feed value has been defined as the combination of the feed nutrient (chemical) composition and digestibility, with consideration to the indigestible portion (Schneider and Flatt, 1975).

Considered the conventional method of digestibility determination, total collection involves measuring total feed (or nutrient) intake (g, DMB) and total fecal output (g, DMB) for each individual subject using the following calculation (Schneider and Flatt, 1975):

$$\text{Nutrient Digestion Coefficient} = 100 \times \frac{Nutrient\ Intake\ (g) - Nutrient\ Output\ (g)}{Nutrient\ Intake\ (g)}$$

This method is time, labor, and resource intensive (Sutton et al., 1977; Van Keulen and Young, 1977), and total fecal output collection requires animals that are adapted to frequent handling and a fecal collection apparatus (Doyle et al., 1994).

In contrast, acid insoluble ash (AIA) is an indigestible, naturally-occurring marker made of inorganic material (silica) in feeds that can also be measured in feces (Agazzi et al., 2011), allowing for apparent digestibility determination using the following calculation (Schneider and Flatt, 1975):

$$\text{Nutrient Digestion Coefficient} = (100 - 100 \times (\frac{Feed\ Indicator\ (\%)\ x\ Fecal\ Indicator\ (\%)}{Fecal\ Nutrient\ (\%) x\ Feed\ Nutrient\ (\%)}))$$

Acid insoluble ash is determined using hydrochloric acid after determination of initial ash concentration (Van Keulen and Young, 1977). While this indirect method also requires a fecal collection apparatus to avoid soil contamination of silica with the fecal sample (Agazzi et al., 2011), it only requires enough fecal sample to accommodate the 20 g dry-sample minimum for analysis.

There have been multiple studies comparing total collection and the AIA method (Sutton et al., 1977; Van Keulen and Young, 1977; Doyle et al., 1994; Agazzi et al., 2011). Sutton et al. (1977) demonstrated no significant difference between apparent digestibility of energy and nitrogen based on method (total collection and AIA), using geldings (n = 4) fed a grain and hay mix (Exp. 1) or pelleted alfalfa and crushed oats (Exp. 3) to meet maintenance requirements. Additionally, a strong correlation between total collection and AIA with r = 0.77 to 0.98 for apparent digestibility of energy, and r = 0.74 to 0.98 for apparent digestibility of nitrogen was demonstrated (Exp. 1, Sutton et al., 1977). The effect of single grab-samples versus composited samples was tested over 4 d, and no significant difference between apparent digestibility of energy and nitrogen based on single grab versus composite samples was found (Exp. 2, Sutton et al., 1977).

Components of particular interest with regards to hindgut digestibility are neutral (NDF) and acid detergent fiber (ADF). Neutral detergent fiber is a collective term referring

to the plant cell wall components of cellulose, hemicellulose, and lignin, while acid detergent fiber is a collective term for cellulose and lignin (Van Soest, 1987). Cellulose and hemicellulose are digestible by microbial enzymes in the rumen or hindgut, while lignin is indigestible (Van Soest, 1987). These components are then fermented into other compounds, such as short-chain fatty acids, that supply energy to the host (Bayer et al., 1998; Donoghue and McKeown, 1999; Schwarz, 2001; Carere et al., 2008). This energy source can supply up to 80% of the daily maintenance energy requirements of the domestic horse (*Equus caballus*; NRC, 2007), and up to 40% for the green iguana (*Iguana iguana*; McBee and McBee, 1982).

Digestibility can be affected by feed particle size alone (Merchen, 1988), and by the combination of NDF concentration and feed particle size (Mertens, 1997). Higher crude fiber (CF) apparent digestibility (aDig, %) of coarsely chopped grass or alfalfa versus the same components finely ground and/or pelleted has been reported in cattle and sheep (Table 5) (Rodrigue and Allen, 1960; Alwash and Thomas, 1971). On the contrary, DM and CF aDig (%) of a chopped composite diet (5.0 mm) fed to juvenile Galapagos tortoises (*Geochelone nigra*, $n = 4$; Hatt et al., 2005), was lower than the DM and NDF aDig (%) of an extruded feed fed to juvenile leopard tortoises (*Stigmochelys pardalis*, $n = 18$; Lickel, 2010), despite comparable nutrient concentration of the diets (Table 5). The contrast in apparent digestibility may be due to the difference in gastrointestinal tract anatomy of ruminant and hindgut-fermenting vertebrates.

Table 5. Mean apparent digestibility (aDig, %) of selected nutrients of hindgut-fermenting and foregut-fermenting vertebrates.

			Mean aDig, %[1]						
Species	**Sample Size (*n*)**	**Diet**	**DM[2]**	**OM[2]**	**CF[2]**	**NDF[2]**	**ADF[2]**	**Cellulose**	**Source**
Stigmochelys pardalis	18	Extruded feed[3]	84.4	85.2	——	76.2	85.0	83.9	Lickel, 2010
Geochelone nigra	4	Composite diet[4]	65.0	67.0	55.0	——	49.0	54.0	Hatt et al., 2005
	2	Grass hay and green leaf lettuce	64.5	66.5	——	56.0	54.0	——	Franz et al., 2011
G. carbonaria	4	Lantana foliage	——	38.0	——	37.0	——	——	Bjorndal et al., 1989
G. denticulata	5	Lantana foliage	——	41.0	——	41.0	——	——	Bjorndal et al., 1989
G. sulcata	7	Grass hay and green leaf lettuce	66.7	68.9	——	65.6	51.6	——	Franz et al., 2011
Testudo graeca	3	Grass hay and green leaf lettuce	91.0	91.7	——	83.7	90.0	——	Franz et al., 2011
T. hermanni	6	Grass hay and green leaf lettuce	87.0	87.3	——	80.8	77.4	——	Franz et al., 2011
Equus caballus	7	"High fat" pelleted diet[5]	63.9	66.0	——	55.8	54.7	——	Schwartz, 2015
	7	"Low fat" pelleted diet[5]	61.6	63.9	——	58.4	57.9	——	Schwartz, 2015
Ovis aries	4	Pelleted grass	——	80.5	78.7	——	——	——	Alwash and Thomas, 1971
	4	Coarsely chopped grass[6]	——	82.7	84.8	——	——	——	Alwash and Thomas, 1971
Bos taurus	8	Coarse-grind alfalfa and concentrate[7]	——	——	34.1	——	——	——	Rodrigue and Allen, 1960
	8	Medium-grind alfalfa and concentrate[7]	——	——	30.8	——	——	39.4	Rodrigue and Allen, 1960
	4	Find-grind alfalfa and concentrate[7]	——	——	22.2	——	——	30.1	Rodrigue and Allen, 1960

[1]All nutrient concentrations are on a dry matter basis (DMB)
[2]DM, dry matter; OM, organic matter; CF, crude fiber; NDF, neutral detergent fiber; ADF, acid detergent fiber
[3]Nutritionally complete, extruded herbivorous tortoise diet fed 7 d per week (Mazuri® 5M21)
[4]Hay (77%), tortoise pellets (15%, Dorswal, Roswal Products, Zurich Switzerland), and apples (8%) chopped to 5.0 mm fed 7 d per week
[5]Commerically available pelleted, herbivorous hindgut-fermenter diet containing a higher (6.9%) or lower (1.7%) inclusion rate of soybean oil
[6]3.0 – 12.0 mm
[7]Two parts alfalfa to one part ground concentrate

After swallowing, digesta of the ruminant animal reaches the reticulum, followed by the rumen where microbial digestion and fermentation occurs (Van Soest, 1994). In order for digesta to leave the rumen, and continue to the omasum, the digesta must pass through the reticulo-omasal orifice (Mertens, 1997). The average diameter of the reticulo-omasal orifice is 1.18 mm (Mertens, 1997), suggesting 1) any particle with a length greater than 1.18 mm requires further degradation (either by rumination or microbial digestion) to pass through the orifice, and continue moving along the gastrointestinal tract, and 2) particles less than 1.18 mm can pass through the orifice undigested by rumen microbes. This may explain the higher apparent digestibility associated with longer-stem forage in cattle and sheep, reported by Rodrigue and Allen (1960) and Alwash and Thomas (1971), respectively. However, this orifice is lacking in hindgut-fermenters, and the digestion of fiber fractions occurs much farther down the gastrointestinal tract. Particles are initially digested via autoenzymatic digestion in the foregut and midgut, leaving the indigestible components (cellulose, hemicellulose, and lignin) to travel to the hindgut. Cellulose and hemicellulose are digested and fermented by hindgut microbes, which may favor smaller particle lengths that are more easily and quickly digested (Bjorndal et al., 1990), since the microbes reside near the end of the gastrointestinal tract. This may lead to higher apparent digestibility of diets comprised primarily of ground or finely chopped ingredients.

The objective of this study was to investigate the apparent digestibility (aDig, %) of dry matter (DM), organic matter (OM), neutral detergent fiber (aNDF), and sequential acid detergent fiber (sADF) of three pelleted diets fed to leopard tortoises (*Stigmochelys pardalis*). One diet was a nutritionally complete, herbivorous tortoise basal diet (CNTRL), and the other two diets were comprised of the same basal diet with added 2.0 or 0.2 mm

length cellulose fiber (further referred to as 2.0 mm and 0.2 mm, respectively). The cellulose fiber lengths were chosen as possible definitions for physically effective neutral detergent fiber (peNDF) for hindgut-fermenting vertebrates. Working from the peNDF definition for dairy cattle (Mertens, 1997), we have defined peNDF for hindgut-fermenting vertebrates as the combined influence of fiber particle size and NDF concentration that promotes hindgut motility as it relates to microbial fermentation of structural carbohydrates. Larger particles move more slowly through the gastrointestinal tract and require longer periods of time for microbial digestion and fermentation (Bjorndal et al., 1990). Increased digestibility may be a result of a slower digesta passage rate. Therefore, we hypothesized that if either cellulose fiber length acted as peNDF, apparent digestibility of all selected nutrients (dry matter (DM), organic matter (OM), neutral detergent fiber (aNDF) and sequential acid detergent fiber (sADF)) would increase, compared to the basal-only (CNTRL) diet. This information may be applicable to the animal feed industry where finely ground ingredients are often used in pelleted and extruded diets designed for herbivorous hindgut-fermenting vertebrates.

5.1 Materials and Methods

5.1.1 Gross Energy

Approximately 25 g of each diet was individually ground using a handheld grinder (IKA® A11 basic, IKA Works, Inc., Wilmington, NC) and recovered into an 18 oz sample bag (Whirl-Pak® B01065, Nasco, Fort Atkinson, WI). The grinder was thoroughly cleaned between each use (diet) with alcohol and dried using acetone. Subsamples (0.5000 to 1.0000 g) of the ground diets were analyzed in duplicate. Samples were first weighed onto weigh paper and pressed into pellets using a Parr pellet press (Parr® Pellet press, Parr

Instrument Company, Moline, IL). Pellets were placed into a tared bomb capsule, and pellet weight was recorded and used for subsequent calculations.

Bomb capsules containing a pellet were placed into the bomb head, and 10 cm of fuse wire was attached. The bomb head was placed and sealed into the bomb cylinder, and a screw cap was attached. Oxygen was added at 30 psi. A bucket was filled with a weighed amount (g) of deionized (DI) water and placed into the bomb calorimeter (Parr® 1241 Automatic Adiabatic Calorimeter, Parr Instrument Company, Moline, IL). The bomb cylinder was placed into the water bucket, and the calorimeter lid was closed and sealed. A thermometer was kept in the surrounding calorimeter jacket and another was lowered into the water bucket once the lid was sealed. Both thermometers recorded respective water temperature throughout the process.

The bomb calorimeter was turned on, and the jacket water temperature was allowed time to equilibrate to the bucket temperature. The thermometers were continually monitored until the temperature readings were equivalent; this indicated that the sample could be ignited. After ignition, the thermometer in the water bucket was checked for a temperature increase indicating that ignition was successful. Water bucket temperature was checked and recorded at 6, 7, and 8 min after ignition to ensure consistent temperature. If all temperatures were consistent, the calorimeter was turned off and the bomb cylinder removed. After releasing the residual air, the bomb screw cap and head were removed. Any remaining wire was removed and recorded. The bomb cylinder, head, and capsule were thoroughly rinsed into a 400 mL glass beaker with DI water to remove all nitric acid (HNO_3) produced in the process. The beaker of washing was placed onto a stir plate (Isotemp® 1110016S, Thermo Fisher Scientific Inc., Asheville, NC) run at approximately

500 RPM, and 4 to 5 drops of methyl orange indicator was added to the beaker. Washings were titrated with sodium carbonate (Na_2CO_3) to neutralize the solution. Beginning and ending amounts (mL) of Na_2CO_3 were recorded. The gross heat of combustion (calories per g) was calculated using initial sample weight (g), bucket temperature at ignition (°C), bucket temperature after 8 min (°C), burned wire (cm) calculated by difference, starting NA_2CO_3 (mL), and ending Na_2CO_3 (mL).

5.1.2 Dry Matter, Organic Matter, Ash, and Acid-Insoluble Ash

Diets and feces were analyzed for dry matter (DM), organic matter (OM), ash, and acid insoluble ash (AIA) in duplicate. Fecal samples were first dried, then ground; diet samples were ground. Individual, frozen fecal samples were removed from their sample bags and placed into pre-weighed, numbered tins. Tins were placed into a 100°C drying oven. Each tin with fecal sample was weighed directly from the drying oven after 24, 48, and 72 h (to ensure a constant weight). Once a constant weight was reached, each fecal sample was removed from the tin and placed into a labeled 55 oz sample bag (Whirl-Pak® B01532WA, Nasco, Fort Atkinson, WI). Dry matter was calculated as the final dry sample weight divided by the original, frozen sample weight, and multiplied by 100 for a percent value. Dried fecal samples were ground through a 2.0 mm screen using a table-top, stainless steel, grinding mill (3383-L60, Thomas Scientific®, Swedesboro, NJ), since all fecal samples were below the 25 g capacity limit. Approximately 50 g of each diet type was ground through a 2.0 mm screen using a variable speed digital ED-5 Wiley Mill (3379-K41, Thomas Scientific®, Swedesboro, NJ). Fecal samples (g) were composited per animal using a series of calculations including adjusted (ADJ) total dry weight (DW) and corresponding percent contribution for each individual fecal sample, as follows:

1) Partial %DM = $\left(\frac{Sample\ DW\ (g)}{Sample\ Fresh\ Weight\ (g)}\right) \times 100$

2) ADJ Partial DM (g) = Sample Fresh Weight (g) × $\frac{Partial\ \%DM}{100}$

3) ADJ Total DM (g) = sum of each individual Sample ADJ Partial DM (g)

4) ADJ %DW Distribution = $\left(\frac{ADJ\ Partial\ DM\ (g)}{ADJ\ Total\ DM\ (g)}\right) \times 100$

5) Composite DW (g) = Total Composite (g) Desired × $\left(\frac{ADJ\ \%DW\ Distribution}{100}\right)$

Clean, dry, wide form 100 mL porcelain crucibles (Coorstek 60138, Coors™, Sigma-Aldrich) were placed into a 100°C forced air drying oven for 2 h prior to use. After 2 h, the crucible was removed and placed into a desiccator for 15 min to cool. Once cooled, the crucible (lid and cup) was weighed. The lid was then removed, the crucible tared, and a subsample (10.0000 to 10.9999 g) of ground diet or feces was weighed into the crucible. All samples were analyzed in duplicate. The crucible with sample was then placed back into the forced air drying oven for 24 h. After 24 h, the crucible was removed from the oven and placed into a desiccator for 1 h to cool. At this time, each crucible with sample was individually weighed.

The crucible was then placed into a muffle furnace (Thermolyne Furnace F30438CM, Thermo Fisher Scientific Inc., Asheville, NC) for approximately 12 h. The muffle furnace was programmed to heat to 600°C within 3 h, maintain this temperature for 2 h, cool to 200°C over 5 h or more, and maintain this reduced temperature until the crucible could be removed. After removing the crucible from the muffle furnace and placing it into a desiccator to cool for 1 h, the crucibles were once again weighed (g), and initial ash (%) was calculated. At this time, OM (DMB) could be calculated as the result of initial ash (%) subtracted from DM (%). Once weighed, the sample was removed from

the crucible and transferred to a tared 250 mL beaker with a metal scraper. The recovered sample amount (g) was weighed, followed by the empty, dirty crucible. Percent recovery of sample was calculated (acceptable range of 90 to 100%). A watch glass was placed onto the beaker with sample to avoid contamination and moisture accumulation.

A 3.15 mm dia, ashless filter paper was weighed and fit onto a 3.15 mm dia vacuum funnel using DI water. The funnel was placed onto a 1,000 mL flask with a spout attached to a vacuum hose. A minimum of 1,000 mL DI water was placed into two 500 mL flasks and heated on a hot plate to boiling. One hundred milliliters of 2M HCl was measured into a 150 mL beaker, then poured into the 250 mL beaker containing sample. The beaker (with watch glass) containing HCl and sample was placed onto a hot plate, and heated to a boil. Once brought to a boil, the solution continued to boil for 5 min. After 5 min, the watch glass was rinsed with room temperature DI water into the beaker, and the solution was poured onto the filter paper in the funnel. Once empty, the beaker was rinsed approximately three times with room temperature DI water to remove as much material as possible. Next, the beaker was rinsed several times with 100 mL of boiling DI water and emptied onto the filter paper. Once rinsed with 700 mL, pH of the filtered material was checked by removing the funnel and catching one drop of material on pH paper. If the pH read 6.0, the procedure could be stopped. If the pH was below 6.0, the material was rinsed with another 100 mL boiling DI water, then re-checked. Once the pH was at 6.0, the filter paper was folded to contain the recovered material, and placed into a previously cooled and weighed high form 100 mL porcelain crucible (Coorstek 60110, Coors™, Sigma-Aldrich). The crucible was placed into a muffle furnace for approximately 12 h (following the same settings as

previously described). When the muffle furnace reached 200°C, the crucible was removed, cooled for 1 h in a desiccator, and weighed.

Amount (g) of AIA sample was calculated as the crucible and sample weight minus the clean, dry crucible weight. Corrected AIA sample weight was calculated as the amount of recovered AIA sample, divided by the percent of recovered, initial ash sample (divided by 100). Percent of AIA (DMB) was calculated as the corrected AIA sample weight divided by the initial ash sample weight, multiplied by 100

5.1.3 Neutral Detergent and Acid Detergent Fiber

Composite fecal samples and ground test diet used for the previously described DM, OM, and AIA procedure were also used for determination of neutral detergent fiber (aNDF) and sequential acid detergent fiber (sADF) content. An analytical balance was used to weigh out 0.4500 to 0.5500 g of sample from each fecal composite and diet type. All samples were analyzed in duplicate. Samples were weighed and processed according to diet type (i.e., all fecal samples from the CNTRL diet, and the diet itself, were analyzed together). Samples were weighed into labeled, pre-weighed filter bags (F58, Ankom Technology, Macedon, NY) of 0.08 mm porosity. Once filled, the filter bags were heat-sealed (Impulse Heat Sealer MP-8, Midwest Pacific) approximately 4 mm from the bag top using setting 6. Filter bags were kept on the sealer with the arm depressed for 4 sec after sealing, and allowed to cool for 5 sec before removing. Additionally, one blank filter bag (no added sample) was also labeled, weighed, and sealed with each process. The blank bag allowed for determination of a dry bag weight correction value.

Once all samples of a diet type were prepared, the filter bags were placed onto bag suspender trays. No more than 3 bags were placed on a single tray, all 9 trays were placed

onto the bag suspender regardless of the number of filter bags being processed, and no bags were placed onto the top tray. The bag suspender was placed into the chamber of the Ankom fiber analyzer (Ankom Fiber Analyzer 200, Ankom Technology, Macedon, NY), and 1,500 mL of neutral detergent solution (Ankom Technology, Macedon, NY) was poured evenly onto the filter bags. Additionally, 4 mL of α-amylase (Ankom Technology, Macedon, NY) was added to the chamber along with 20 g of sodium sulfite (Ankom Technology, Macedon, NY). The chamber was closed and sealed, and heat and agitation were turned on for 75 min.

After 75 min, the chamber was slowly vented and released of all liquid. After complete venting, the filter bags were rinsed by opening the chamber, closing the valve, and adding 1,900 mL of hot DI water to the chamber along with 4 mL of amylase. The chamber was closed and sealed, and heat and agitation were turned on for 5 min. This rinsing process was repeated 2 more times: the second time with the addition of α-amylase, and the third time without. Once completely rinsed, the filter bags were removed from the trays, excess water was removed from the bags by gently shaking, and the bags soaked in acetone for 5 min. The bags were then placed onto a tray and dried for 24 h in a chemical hood. The tray was then moved to a forced-air drying oven (100°C) for 24 h. After 24 h, the bags were placed into a beaker in a desiccator, and cooled for 1 h. After cooling, the bags were individually weighed. Neutral detergent fiber concentration (%) was calculated as (Ankom, 2015):

$$\%\text{aNDF (DMB)}: \left(\left(\frac{100 \times [W3-(W1 \times C1)]}{W2}\right) \times \%\text{DM}\right)$$

Where, W1 = bag tare weight (g)

W2 = initial sample weight (g)

W3 = dried weight of bag with sample (g)

C1 = blank bag correction (final dry weight (g) / original bag weight (g))

Once the NDF analysis was complete, the filter bags were once again placed on the trays and the trays were stacked on the bag suspender. The suspender was placed into the chamber, and 1,500 mL of acid detergent solution (Ankom Technology, Macedon, NY) was added to the chamber. The chamber was closed and sealed, and heat and agitation were turned on for 60 min. After 60 min, the chamber was vented as previously described. Once empty of all liquid, the valve was closed and 1,900 mL of hot DI water was added to the chamber for the first rinse. After a 5 min rinse, two more rinses, following the same process, were completed. The filter bags were once again soaked in acetone for 5 min, transferred to a tray, and dried in a chemical hood for 24 h. The tray was then transferred to a drying oven for 24 h, followed by cooling of the bags in a desiccator for 1 h, and weighing each individual bag. Sequential acid detergent fiber concentration (%, DMB) was calculated using the same method described for %aNDF (DMB) (Ankom, 2014).

5.1.4 Digestibility Calculations

Digestibility of DM, OM, aNDF, and sADF was calculated for each individual animal (n = 13) using methods described by Schneider and Flatt, 1975. In each applicable equation, "indicator" refers to %AIA.

1) Digestibility of DM, OM, aNDF, and sADF using total collection:

$$\text{Nutrient Digestion Coefficient} = \left(100 \times \left(\frac{Nutrient\ Intake\ (g) - Nutrient\ Output\ (g)}{Nutrient\ Intake\ (g)}\right)\right)$$

2) Digestibility of DM using AIA:

$$\text{DM Digestion Coefficient} = \left(100 - 100 \times \left(\frac{Feed\ Indicator\ (\%)}{Fecal\ Indicator\ (\%)}\right)\right)$$

3) Digestibility of OM, aNDF, and sADF using AIA:

$$\text{Nutrient Digestion Coefficient} = \left(100 - 100 \times \left(\frac{Feed\ Indicator\ (\%)}{Fecal\ Nutrient\ (\%)} \div \frac{Fecal\ Indicator\ (\%)}{Feed\ Nutrient\ (\%)}\right)\right)$$

5.1.5 Statistical Analysis

Of the 16 tortoises in the experimental design, one tortoise was removed due to duplicate sample values over the acceptable threshold (CV ≤ 0.05), and two additional tortoises were removed due to insufficient sample amount to perform the acid insoluble ash (AIA) procedure in duplicate. A repeated measure analysis of variance (ANOVA), where individual tortoise was the repeated measure, was used to determine if a significant difference was present between: 1) digestibility determination method (total collection and AIA) for each nutrient (DM, OM, aNDF, and sADF), 2) nutrient apparent digestibility (aDig, %) by diet (CNTRL, 2.0 mm, and 0.2 mm), and 3) an interaction between method and diet. Additionally, if a significant difference was found based on diet, Tukey's pairwise comparison was used to investigate if a significant difference was present among each possible pair of digestibility by diet. All analyses used a significance level of $P < 0.05$ in JMP Pro 12 (SAS Institute Inc., Cary, NC).

5.2 Results

5.2.1 Selected Nutrient Analysis

Measured GE for all test diets was similar: CNTRL, 18.62 kJ/g; 2.0 mm, 18.74 kJ/g; and 0.2 mm, 17.87 kJ/g. Dry matter (DM) was similar for all 3 diets; most other nutrients were similar between the cellulose-added diets (2.0 and 0.2 mm), but different between the cellulose-added diets and the control diet (CNTRL) (Table 6). Neutral detergent fiber (aNDF) was substantially lower in the 0.2 mm diet than formulated and expected; it was more similar to the CNTRL diet than the 2.0 mm diet. However, sADF was similar between the 2.0 and 0.2 mm diets.

Table 6. Selected nutrients of three pelleted diets fed to leopard tortoises (*Stigmochelys pardalis*).

Nutrient[1]	CNTRL[3]	2.0 mm[4]	0.2 mm[5]
DM[2], %	90.85	90.96	89.58
OM[2], %	91.88	93.14	93.16
Ash, %	8.12	6.86	6.84
AIA[2], %	0.35	0.31	0.28
aNDF[2], %	29.25	39.17	32.98
sADF[2], %	14.57	25.42	24.63
GE (kJ/g)	18.62	18.74	17.87

[1]All nutrient concentrations are on a dry matter basis (DMB), except DM
[2]DM, dry matter; OM, organic matter; AIA, acid insoluble ash; aNDF, α-amylase treated neutral detergent fiber; sADF, sequential acid detergent fiber; GE, gross energy
[3]Nutritionally complete, herbivorous tortoise diet base (Mazuri® 5M21)
[4]Base diet and 2.0 mm length cellulose fiber (84% and 16%, respectively)
[5]Base diet and 0.2 mm length cellulose fiber (84% and 16%, respectively)

5.2.2 Apparent Digestibility

Digestibility of DM, OM, aNDF, and sADF based on total fecal collection and acid insoluble ash (AIA) marker of three pelleted diets fed to leopard tortoises is reported below (Table 7). There was no interaction between diet and method for any nutrient ($P > 0.05$). Dry matter and organic matter digestibilities were not different between diet ($P = 0.4932$, $P = 0.5659$, respectively), nor between method ($P = 0.5016$, $P = 0.6899$, respectively). Neutral (aNDF) and sequential acid detergent fiber (sADF) digestibilities were different based only on diet ($P = 0.0325$ and $P = 0.0004$, respectively). Neutral detergent fiber digestibility of the 0.2 mm diet was significantly different from the CNTRL ($P = 0.0373$), but not different from the 2.0 mm diet ($P = 0.9611$), while aNDF digestibility of the 2.0 mm diet was not different from the CNTRL ($P = 0.0723$) (Table 8). Sequential acid detergent fiber digestibility of the 0.2 and 2.0 mm diets was also different from the CNTRL ($P = 0.0003$, $P = 0.0039$, respectively), but not different between one another ($P = 0.3552$) (Table 8).

Table 7. Mean apparent digestibility (aDig, % ± SD) of selected nutrients of three pelleted diets fed to leopard tortoises (*Stigmochelys pardalis*) based on total fecal collection and acid insoluble ash (AIA) marker methods.

	Mean aDig, %							
Diet	**DM[2]**		**OM[2]**		**aNDF[2]**		**sADF[2]**	
	Total	**AIA**	**Total**	**AIA**	**Total**	**AIA**	**Total**	**AIA**
CNTRL (n = 4)[3]	87.45	88.80	89.17	89.64	81.86	83.36	81.31	82.85
	(± 2.20)	(± 1.31)	(± 1.46)	(± 1.15)	(± 2.55)	(± 1.63)	(± 2.66)	(± 1.72)
2.0 mm (n = 4)[3]	88.85	88.32	90.26	88.91	86.86	86.25	88.36	88.93
	(± 2.29)	(± 1.49)	(± 2.28)	(± 1.51)	(± 2.95)	(± 2.25)	(± 2.95)	(± 3.53)
0.2 mm (n = 5)[3]	89.14	89.99	89.92	90.73	86.32	87.59	90.28	91.18
	(± 2.24)	(± 2.23)	(± 2.13)	(± 2.03)	(± 3.18)	(± 1.90)	(± 2.25)	(± 1.27)
P-value	0.4932[a]		0.5659[a]		0.0353[a]		0.0004[a]	
P-value	0.5016[b]		0.6899[b]		0.4820[b]		0.3436[b]	

[1]All digestibilities are on a dry matter basis (DMB)

[2]DM, dry matter; OM, organic matter; AIA, acid insoluble ash; aNDF, α-amylase treated neutral detergent fiber; sADF, sequential acid detergent fiber

[3]Nutritionally complete, herbivorous tortoise diet base (Mazuri® 5M21) (CNTRL); tortoise diet with added 2.0 (2.0 mm) and 0.2 (0.2 mm) mm length cellulose fiber, pelleted (84% and 16%, respectively)

[a]Significance of diet using pooled total and AIA aDig (%) values at $P < 0.05$

[b]Significance of method at $P < 0.05$

Table 8. Mean apparent fiber fraction digestibility (% and g, ± SD) of leopard tortoises (*Stigmochelys pardalis*) fed one of three pelleted diets, exclusively.

	Mean aDig			
Diet[2]	**aNDF[1]**		**sADF[1]**	
	%	**g**	**%**	**g**
CNTRL (*n* = 4)	82.61 (± 2.14)[a]	284.69 (± 23.85)	82.08 (± 2.23)[a]	140.87 (± 12.04)
2.0 mm (*n* = 6)	86.55 (± 2.49)[a,b]	431.54 (± 35.52)	88.64 (± 3.08)[b]	284.77 (± 20.87)
0.2 mm (*n* = 6)	86.95 (± 2.57)[b]	356.85 (± 18.85)	90.73 (± 1.80)[b]	278.79 (± 13.58)

All digestibilities are on a dry matter basis (DMB)
[1]aNDF, α-amylase treated neutral detergent fiber; sADF, sequential acid detergent fiber
[2]Nutrionally complete, herbivorous tortoise diet (Mazuri® 5M21) (CNTRL); tortoise diet with added 2.0 (2.0 mm) or 0.2 (0.2 mm) mm length cellulose fiber, pelleted (84% and 16%, respectively)
Different letters indicate significant differences ($P < 0.05$) of aDig, %

5.3 Discussion

5.3.1 Selected Nutrient Analysis

Measured GE of the CNTRL diet was slightly higher than previously measured for the same formula in an extruded form (18.18 kJ/g; Lickel, 2010). Since all diets in this study, and that of Lickel (2010), were ground and pelleted using the same procedure for GE determination via bomb calorimetry, the original form of the diet (pelleted versus extruded) should not be a factor in the difference. It was expected that GE of the cellulose-added diets (2.0 and 0.2 mm) would be more similar to one another than to the CNTRL due to their expected nutrient concentration similarities. However, the 2.0 mm and CNTRL diets were more similar.

The DM, OM, ash, AIA, aNDF, and sADF of the pelleted CNTRL diet is similar to that determined for the same formula as an extruded diet (Lickel, 2010), although sADF was slightly lower than expected in the CNTRL diet, and lower than that determined by

Lickel (2010) (Table 9). This may be due to typical variation in raw ingredient composition or human error during analysis, although duplicate results were very consistent.

Table 9. Selected nutrient analysis of a nutritionally complete, herbivorous tortoise diet, pelleted and extruded.

Nutrient[1]	CNTRL[3]	Extruded Tortoise Diet[4]
DM[2], %	90.85	92.6
OM[2], %	91.88	91.9
Ash, %	8.12	8.1
AIA[2], %	0.35	—
aNDF[2], %	29.25	29.9
sADF[2], %	14.57	18.1

[1]All nutrient concentrations are on a dry matter basis (DMB), except DM
[2]DM, dry matter; OM, organic matter; AIA, acid insoluble ash; aNDF, α-amylase treated neutral detergent fiber; sADF, sequential acid detergent fiber
[3]Nutritionally complete, herbivorous tortoise diet, pelleted (Mazuri® 5M21)
[4]Nutritionally complete, herbivorous tortoise diet (Mazuri® 5M21; Lickel, 2010)

The selected nutrient analysis of the 2.0 mm and 0.2 mm diets are similar with regards to DM, OM, ash, AIA, and sADF (Table 9). Measured aNDF was similar between the 2.0 mm diet and the model diet (Mazuri® 5E5L), but aNDF was, surprisingly, lower in the 0.2 mm diet (Table 10). This difference is believed to be due to error during analysis. Results were very consistent among duplicates of the CNTRL and 2.0 mm diets, but highly variable for duplicates of the 0.2 mm diet, ranging from 32% to 37% aNDF.

Table 10. Selected nutrient analysis of two pelleted, cellulose-added diets and a similar, commercially available extruded, herbivorous tortoise diet.

Nutrient[1]	2.0 mm[3]	0.2 mm[4]	Extruded Tortoise Diet[5]
DM[2], %	90.96	89.58	88.0
OM[2], %	93.14	93.16	92.2
Ash, %	6.86	6.84	7.8
AIA[2], %	0.31	0.28	—
aNDF[2], %	39.17	32.98	41.0
sADF[2], %	25.42	24.63	25.0

[1]All nutrient concentrations are on a dry matter basis (DMB), except DM
[2]DM, dry matter; OM, organic matter; AIA, acid insoluble ash; aNDF, α-amylase treated neutral detergent fiber; sADF, sequential acid detergent fiber
[3]Nutritionally complete, herbivorous tortoise diet base (Mazuri® 5M21) and 2.0 mm length cellulose fiber, pelleted (84% and 16%, respectively)
[4]Nutritionally complete, herbivorous tortoise diet base (Mazuri® 5M21) and 0.2 mm length cellulose fiber, pelleted (84% and 16%, respectively)
[5]Nutritionally complete, low starch herbivorous tortoise diet, (Mazuri® 5E5L; Mazuri, 2016)

5.3.2 Apparent Digestibility

Based on our hypothesis that aDig (%) of all nutrients would increase with the cellulose-added diets (2.0 mm, 0.2 mm) if either fiber length acted as peNDF, our results are conflicting regarding the action of particle length and determination of a peNDF threshold for leopard tortoises. No difference in DM and OM digestibility across diets, coupled with differences in aNDF and sADF digestibility, suggests a shift in the source of OM that was digested in the CNTRL diet compared to the cellulose-added diets. Apparent digestibility of aNDF and sADF for the CNTRL diet was similar to that reported by Lickel (2010) for the same feed in an extruded form. These same digestibilities were both significantly greater for the 0.2 mm diet, with sADF significantly greater for the 2.0 mm diet, corroborating the ideas of Merchen (1988) and Mertens (1997) that fiber particle size affects fiber digestibility. Therefore, these results call into question the previously described definition of peNDF for hindgut-fermenting vertebrates. If the idea of peNDF

for hindgut-fermenters is a resulting slower digesta passage rate to allow more time for microbial digestion and fermentation, then this may have been accomplished as suggested by increased aNDF and sADF digestibilities of the cellulose-added diets. Furthermore, if there was, indeed, greater digestibility of the cellulose (represented in aNDF and sADF) in the treatment diets, then significant recovery of those particle lengths in the feces would not be expected, as previously postulated. Future studies should consider measuring digesta passage rate alongside digestibility with regards to fiber particle length.

The digestibility of OM and aNDF in our captive *S. pardalis* colony are similar to those reported by Hailey (1997) of OM and holocellulose (cellulose and hemicellulose) aDig (%) by *S. pardalis* on kale, but much less than *S. pardalis* on grass (Table 11). Although holocellulose does not include lignin, as NDF does, lignin is typically found in much lesser concentrations in grasses compared to woody plants. Additionally, all three of the diets used in this study contain easily digestible ingredients (i.e., ground soybean hulls, ground corn) that may have contributed to the increased aDig (%) compared to *S. pardalis* fed grass. As Mertens (1997) defined peNDF for dairy cattle, both fiber (NDF) concentration and fiber particle length are important aspects to fiber digestibility. With respect to the added cellulose, although the aNDF concentrations (%) for the 2.0 and 0.2 mm diets were greater than the CNTRL, cellulose was listed in third place of the ingredient list, behind ground soybean hulls and ground corn, for the test diets. This indicates that ground soybean hulls and ground corn were both in greater amounts in the diets than the cellulose. The cell content concentration (%) of DM in each of the test diets was 78.9% (CNTRL), 67.7% (2.0 mm), and 73.9% (0.2 mm); calculated as:

[%DM – (%aNDF - %ash)].

Table 11. Concentration (%) of organic matter (OM), holocellulose, and neutral detergent fiber (aNDF) in diets and their accompanying apparent digestibilties (aDig, %) when fed to leopard tortoises (*Stigmochelys pardalis*).

Diet	Nutrient[1]	Diet, %[2]	aDig, %[2]	Source
kale (Brassica)	OM	86.0	82.2	Hailey, 1997
	Holocellulose	27.1	88.8	Hailey, 1997
grass (Lolium)	OM	83.3	63.9	Hailey, 1997
	Holocellulose	27.1	62.7	Hailey, 1997
CNTRL[3]	OM	91.9	89.2	Current study
	aNDF	29.3	81.9	Current study
2.0 mm[3]	OM	93.1	90.3	Current study
	aNDF	39.2	86.9	Current study
0.2 mm[3]	OM	93.2	89.9	Current study
	aNDF	33.0	86.3	Current study

[1]OM, organic matter; holocellulose, cellulose and hemicellulose; aNDF, α-amylase treated neutral detergent fiber

[2]All nutrients (diet, %) and apparent digestibilities (aDig, %) are on a dry matter basis (DMB)

[3]Nutritionally complete, herbivorous tortoise diet base (Mazuri® 5M21); tortoise diet with added 2.0 (2.0 mm) and 0.2 (0.2 mm) mm length cellulose fiber, pelleted (84% and 16%, respectively)

6. FECAL MICROBIOME

Microbiota, microflora, and microbes reference the communities of microorganisms inhabiting a host niche (Hooper et al, 2002). In contrast, microbiome references the genes rooted in the genome of the microorganisms that make up the communities (Hooper et al, 2002; Ley et al, 2008). Microbes are bacteria, protozoa, and fungi that perform a variety of functions, including digestion and fermentation in the vertebrate gastrointestinal tract (Klasing, 2005; Stevens and Hume, 1998 and 2004). Microbes and vertebrates coevolved, establishing a persisting symbiotic relationship exemplified in herbivorous animals (Kostic et al, 2013; Ley et al, 2008).

Microbes are responsible for digesting complex polysaccharides that are otherwise unavailable to animal hosts (Bennett et al, 2013; Edwards, 1997; Klasing, 2005; Ullrey et al, 1997; Wrong et al, 1981). Microbes digest cellulose and hemicellulose, which are measured, along with lignin, as neutral detergent fiber (NDF). Along with short-chain fatty acids (SCFAs), microbial fermentation also produces protein that can contribute to host protein metabolism (Atlas and Bartha, 1998; Arthur et al, 2014), and B vitamins and vitamin K that can be used for cell metabolism (Atlas and Bartha, 1998; Hooper et al, 2002).

The colonization of gastrointestinal microbes was essential for evolution towards an herbivorous diet (Hong et al, 2011; Ley et al, 2008). While herbivores comprise a majority of mammalian biomass, they represent only a small portion of reptilian species. Of the approximately 100 recognized reptilian herbivores, tortoises represent nearly half (Stevens and Hume, 1998). Nearly all tortoises are hindgut-fermenters utilizing enlarged regions of the large intestine (colon and cecum) for microbial fermentative digestion of

plant material (Stevens and Hume, 1998; Wrong et al, 1981). The outcomes of many gastrointestinal tract microbial studies have validated the density of bacterial microbes inhabiting the large intestine of multiple vertebrate species, reporting 10^8 to 10^{12} microbes per mL of intestinal contents, representing up to 400 species (Atlas and Bartha, 1998; Stevens and Hume, 2004; Leser and Mølbak, 2009). Microbiota (i.e., phylum, order, class, etc.) are most often reported as percentages of operational taxonomic units (OTUs). An OTU is a cluster of 16S rRNA sequences that are classified based on their similarity with other sequences within a data set (Schloss and Westcott, 2011).

Within herbivorous, hindgut-fermenting reptiles, fecal microbiota is dominated by the phylum Firmicutes (Table 12), which consists of species with low molar fractions of guanine and cytosine in their DNA, some with spore-forming capability (Galperin, 2013). Fecal microbiota of Galapagos land iguanas (*Conolophus subscristatus*), Galapagos marine iguanas (*Amblyrhynchus cristatus*), green iguanas (*Iguana iguana*), and Galapagos tortoises (*Geochelone nigra*) have demonstrated Firmicutes ranging from 63.9 to 81.1% of OTUs (Hong et al., 2011). The next abundant phylum in these individuals was Bacteroidetes, although it paled in comparison to Firmicutes, ranging from 4.2 to 10.1% of OTUs (Hong et al., 2011). Conversely, Yuan et al. (2015) identified Firmicutes and Bacteroidetes in almost identical proportions from fecal microbiota of gopher tortoises (*Gopherus polyphemus*), reported as 36.0 and 36.5% of OTUs, respectively. Within some of these phyla, more specific identifications have been made, and are discussed in more detail.

Table 12. Fecal archaea and bacterial microbial OTU contributions of free-ranging herbivorous, hindgut-fermenting reptiles.

Species	*n*	Kingdom	Subdivision[1]	% of Total OTUs	Source
Conolophus subcristatus	10	Archaea	Unclassified	0.1 – 0.2	Hong et al., 2011
		Bacteria	*Firmicutes*	*63.9*	
C. pallidus			Clostridia	~50.0	
	6		*Bacteroidetes*	*4.2*	
			Unclassified	26.6	
Amblyrhynchus cristatus	25	Bacteria	*Firmicutes*	*75.1*	Hong et al., 2011
			Clostridia	~50.0	
			Bacteroidetes	*8.2*	
			Unclassified	14.5	
Iguana iguana	2	Archaea	Unclassified	0.1 – 0.2	Hong et al., 2011
		Bacteria	*Firmicutes*	*74.0*	
			Clostridia	~50.0	
			Bacteroidetes	*10.1*	
			Unclassified	10.1	
Geochelone nigra	4	Archaea	Unclassified	0.1 – 0.2	Hong et al., 2011
		Bacteria	*Firmicutes*	*81.1*	
			Clostridia	~50.0	
			Bacteroidetes	*4.4*	
			Unclassified	10.4	
Gopherus polyphemus	46	Archaea	Methanoshaera	1.0	Yuan et al., 2015
		Bacteria	*Firmicutes*	*36.0*	
			Clostridia	97.0	
			Erysipelotrichi	0.80	
			Bacilli	0.05	
			Bacteroidetes	*36.5*	
			Bacteroidales	79.7	
G. polyphemus		Bacteria	*Firmicutes*	*59.7*	Gaillard, 2014
			Bacteroidetes	*15.9*	
			Proteobacteria	*15.4*	

[1]Italics indicates phylum; indentations following italics indicates further identification within the preceding phylum

6.1 Microbial Functions

6.1.1 Amylolytic

Starch, the carbohydrate-storage mechanism of plants, consists of α-1,4 and α-1,6 linked amylose and amylopectin molecules that can be digested by amylase enzymes secreted by the host animal's small intestine (Van Soest, 1987). Starches that resist degradation in the small intestine are sources of energy for microbes (Andreesen et al., 1989), and for the host via fermentation (Van Soest, 1987). However, starches are more rapidly fermented, since they are easily digested by host enzymes into glucose molecules (NRC, 2007). Rapid fermentation may result in hindgut dysfunction (NRC, 2007). The hydrolysis of starch components (amylose, amylopectin, and glycogen) yields glucose and maltose (Sharp et al., 1989); maltose can be further digested, while glucose can enter the fermentation cycle and produce SCFAs. Members of Bacilli have been identified as amylolytic, and although unspecified, Bacilli has been identified in the fecal microbiome of *G. polyphemus* (Yuan et al., 2015).

6.1.2 Cellulolytic and Hemicellulolytic

Cellulose and hemicellulose consist of β-1,4 linked glucose molecules that cannot be digested by the host animal's enzymes (Van Soest, 1987; Schwarz, 2001). As previously described, the highly studied *Clostridium thermocellum* has been the model species for understanding the microbial enzyme complex for cellulose digestion, termed the cellulosome (Lamed and Bayer, 1986; Bayer et al., 1998; Doi and Kosugi, 2004). The majority of cellulolytic species are also hemicellulolytic (Yokoyama and Johnson, 1988). Short-chain fatty acids produced through microbial fermentation of cellulose do not provide energy to the microbe, but they can be used by microbes to synthesize other

products, such as amino acids and long-chain fatty acids for microbial growth (Van Soest, 1987). Ethanol, a by-product of cellulose fermentation, can also be used as a substrate for acetate production (Andreesen et al., 1989). The cellulolytic Clostridia families Ruminococcaceae, Lachnospiraceae, Christensenellaceae, Clostridiaceae, Clostridiaceae and Synthrophomonadaceae, as well as the Bacilli order Bacteroidiales (Bacteroidetes) have been identified in the fecal microbiome of *G. polyphemus* (Yuan et al., 2015).

6.1.3 Pectinolytic

Pectins consist of the simple (monosaccharide) sugars, arabinose and galactose, and the sugar acid, galacturonic acid (Van Soest, 1987). Pectin is present as a "cementing" property in the plant cell wall (Van Soest, 1987). Although these sugars are α-1,4 linked, host animal enzymes are not capable of completely digesting the linkages due to the presence of an axial bond on carbon 4 and a rhamnose unit with an α-1,2 linkage (Van Soest, 1987, 1994). The components of pectin can be fermented into SCFAs (Van Soest, 1987), providing energy to the host. Although not reported in the fecal microbiomes of herbivorous, hindgut-fermenting reptiles, Clostridia species have been identified as pectinolytic (Andreesen et al., 1989).

6.1.4 Proteolytic

Amino acids that resist degradation in the small intestine are sources of carbon, nitrogen, and energy for microbes (Andreesen et al., 1989). Cellulolytic species rely on proteolytic species to produce volatile fatty acids, particularly n-valeric, isovaleric, isobutyric, and 2-methlybutyric acid, from deamination of amino acids (Yokoyama and Johnson, 1988). As electron donors, amino acids can supply carbon and nitrogen

(Andreesen et al., 1989) that can be utilized in the fermentation of other products, or for microbial protein synthesis (Shirley, 1986).

Additionally, proteolytic species aid in nitrogen metabolism from the catabolism of proteins (Kormelink et al., 2012), most likely in the form of microbial protein synthesis. Members of Bacilli share an important role as proteolytic species. Amino acids are utilized by Bacilli to produce microbial protein, SCFAs, ammonia, carbon dioxide, and methane (Shirley, 1986). A source of nitrogen from amino acids allows for the production of ammonia (NH_3), a required precursor for microbial protein synthesis (Shirley, 1986). Evidence of microbial protein in the tissues of herbivorous, hindgut-fermenting green sea turtles (*Chelonia mydas*) via stable carbon-isotope analysis indicate a contribution of essential amino acids from gut microbes to the host (Arthur et al., 2014). The importance of this contribution has yet to be demonstrated.

6.1.5 Methanogenic

The production of methane (the most reduced form of carbon) by archaea, is a distinguishing characteristic from the other domains (bacteria, eucarya) (Danson et al., 2007). Methanogens represent the largest group of archaea (Danson et al., 2007). These microbes utilize end-products and by-products from other bacteria and fungi, such as acetate, carbon dioxide, and hydrogen for methane production (Danson et al., 2007). Although each order of methanogens appears to have a preferential source for methane production, most have the ability to reduce carbon dioxide using several reductants, including electrons from hydrogen oxidation, formate production, and secondary alcohols (by-products) (Danson et al., 2007). Although present, methane production has been demonstrated to be much less in the cecum of hindgut-fermenting rats, compared to the

rumen of foregut-fermenting cattle, due to a higher affinity for the use of H_2 to reduce CO_2 into acetate (Miller and Wolin, 1979). This may be the case for other hindgut-fermenting species. Methanogens have been identified in the fecal microbiome of *G. polyphemus*, including *Methanobrevibacter* and *Methanosphaera* (Methanobacteriaceae, Methanobacteriales), *Methanospirillum* (Methanospirillaceae, Methanomicrobiales), and Methanocorpusculaceae (Methanomicrobiales) (Yuan et al., 2015).

6.1.6 Saccharolytic

Recognized as an ability of essentially all organisms of all three microbial domains (archaea, bacteria, eucarya), microbes have the ability to metabolize glucose and other simple sugars (Danson et al., 2007). Saccharolytic is, for all intents and purposes, an all-encompassing term for amylolytic and cellulolytic organisms. However, it refers to the metabolism of simple sugars, without considering any bacterial digestion that may have taken place in order to release simple sugars (Miller and Wolin, 1979). The saccharolytic archaea class, Thermoplasmata, has been identified in the fecal microbiome of *G. polyphemus* (Yuan et al., 2015).

6.1.7 Xylanolytic

Recent research into other capabilities of Bacteroidetes reveal xylan-degrading enzymes (Dodd et al., 2011). Xylan is a β-1,4 linked linear chain of xylose (sugar) (Van Soest, 1987; Dodd et al., 2011) found in the plant cell wall. Due to the β-linkages, xylan cannot be digested by the host animal's own enzymes. Fermentation of monosaccharides from xylan degradation may add to the supply of SCFAs for host energy (Dodd et al., 2011), although more research is needed to confirm this hypothesis.

The objective of this study was to characterize the fecal microbiome of leopard tortoises fed one of three nutritionally complete, herbivorous tortoise diets that varied in the inclusion of cellulose fiber at either 2.0 or 0.2 mm in length, in order to identify changes in the microbiome due to dietary (insoluble fiber) influence.

6.2 Materials and Methods

6.2.1 Fecal DNA Isolation

Deoxyribose nucleic acid (DNA) was isolated from frozen fecal samples (n = 16) using the MO BIO Powersoil® DNA Isolation Kit (12888-50, MO BIO Laboratories, Inc., Carlsbad, CA). Frozen fecal samples were removed from storage and 0.25 g was immediately subsampled using an analytical balance, and transferred to a Powersoil® bead tube containing 750 µL of guanidine thiocyanate and garnet buffer solution. The bead tube was vortexed (E193271, Analog Mini Vortexer, Fisher Scientific, Waltham, MA) for 5 sec on speed "7" to mix the feces and solution, dissolving humic acids and protecting nucleic acids from degradation (Powersoil® DNA Isolation Kit Instruction Manual). Using calibrated pipettes (Finnpipette®, Fisherbrand™, Waltham, MA), 60 µL of a proprietary surfactant (C1) was added to the bead tube for complete cell lysis, and the bead tube was vortexed at maximum speed for 10 min, then centrifuged (Centrifuge 5418, Eppendorf, Hauppauge, NY) for 30 sec. All centrifuging occurred at room temperature at a speed of 10,000 x g. Resulting supernatant (~ 400 to 500 µL) was transferred to a clean 2 mL collection tube. Two hundred and fifty microliters of a proprietary protein precipitant (C2) was added to the collection tube, and the tube was vortexed for 5 sec, then incubated at 4°C using an insulated micro-tube holder in a temperature-controlled refrigerator for 5 min. After incubation, the tube was centrifuged for 1 min. Resulting supernatant (~ 600 µL) was

transferred to a clean 2 mL collection tube. Two hundred microliters of an inhibitor removal compound (C3) was added to the collection tube. Vortexing, incubation, and centrifuging occurred following the above parameters.

Resulting supernatant (~ 750 µL) was transferred to a clean 2 mL collection tube. Twelve hundred microliters of a proprietary chaotropic agent (C4) was added to the collection tube, and the tube was vortexed for 5 sec. Six hundred and seventy-five microliters of supernatant was loaded onto a spin filter in a clean 2 mL collection tube, and centrifuged for 1 min. After discarding the flow through, an additional 675 µL of supernatant was again loaded onto the same spin filter and centrifuged at the same parameters. Lastly, the remaining supernatant was loaded onto the same spin filter and centrifuged a third time. Five hundred microliters of ethanol (C5) was added to the spin filter tube containing the filtered supernatant, and the tube was centrifuged for 30 sec. Flow through from the spin filter tube was discarded, and the tube was centrifuged at the above parameters for an additional minute. The spin filter was removed, and transferred to a clean 2 mL collection tube. One hundred microliters of elution buffer (C6) was added directly to the filter, and centrifuged for 30 sec. The spin filter was removed from the collection tube, and tubes were stored at -80°C until they were sent out for DNA analysis.

6.2.2 DNA Sequencing

The isolated DNA samples were packed into an insulated cooler with ice packs, and sent (next-day delivery) to the Mr. DNA lab in Shallowater, Texas. The Mr. DNA lab performed 16S rRNA gene (V4 variable region PCR primers 515/806) analysis to identify microbial (bacterial) species from the isolated DNA. The 16s rRNA gene contains highly variable and conserved regions (Amor and Vaughan, 2006). Differences in the region

sequences are used to determine bacterial species (Amor and Vaughan, 2006). The process involves a polymerase chain reaction (PCR)-mediated amplification of the 16S rRNA genes and subsequent cloning into *Escherichia coli* for individual gene segregation (Amor and Vaughan, 2006). The genes can then be compared to known, catalogued gene sequences for identification.

At the Mr. DNA lab, a HotStarTaq Plus Master Mix Kit using a single-step 30 cycle PCR with an Ion Torrent Personal Genome Machine (PGM) (following manufacturer guidelines) was employed. Each fecal DNA sample was incubated at 94°C for 3 min, followed by 28 cycles of incubation at 94°C for 30 sec. This was followed by incubation at 53°C for 40 sec, incubation at 72°C for 1 min, and elongation at 72°C for 5 min. At this time, the 16S rRNA sequences had been isolated.

Next, the Mr. DNA lab used a proprietary analysis pipeline to analyse the sequences. Barcodes, primers, ambiguous base calls, homopolymer runs greater than 6 base pairs, and base pairs less than 150 were removed from the sequences. Common errors due to high through-put sequencing were removed, OTUs were generated using 97% similarity, and chimeras removed. Lastly, the OTUs were classified (taxonomically) using a comparison with BLASTn (Nucleotide Basic Local Alignment Search Tool, U.S. National Library of Medicine) classifications, Greengenes (Lawrence Berkley National Laboratory), National Centre for Biotechnology Information (NCBI), and Ribosomal Database Project II (RDPII) databases.

6.2.3 Statistical Analysis

We received isolated gene sequences from the Mr. DNA lab, and analyzed the results using Plymouth Routines In Multivariate Ecological Research (PRIMER) version

5.2.9 (PRIMER-E Ltd, 2009, United Kingdom) to identify differences among the fecal microbial DNA profiles based on diet. Diversity indices of transformed (√) raw data were used to determine the normalized numbers of different phyla or genera in samples (J'), and how evenly the abundance of phyla or genera were spread in samples (H') (Heip et al., 1998). These values were tested for significant differences at $P < 0.05$ based on diet using an analysis of variance (ANOVA) in JMP Pro 12 (SAS Institute Inc., Cary, NC). If a significant difference in the ANOVA was found, Tukey's pairwise comparisons were used to test for significant differences between each possible pair of diets. Using PRIMER, similarities of bacterial phyla and genera between diets (ANOSIM), and similarity among bacterial phyla and genera of individual tortoises by diet (SIMPER) were determined.

6.3 Results

Total gene sequences were averaged: 39,479 for the CNTRL diet ($n = 4$), 37,457 for the 2.0 mm diet ($n = 6$), and 41,661 for the 0.2 mm diet ($n = 6$). Similarity of total OTUs between diet was tested (ANOSIM) with and without unidentified sequences ($P = 0.002$ and $P = 0.004$, respectively). There were no differences in diversity indices (J' and H') of total OTUs by diet, with and without unidentified sequences (Table 13). Due to significance with and without the unidentified sequences, unidentified sequences were removed from subsequent analyses.

Table 13. Diversity indices (± standard deviation) of fecal bacterial operational taxonomic units (OTU) with the number of total and unidentified gene sequences for leopard tortoises (*Stigmochelys pardalis*) fed one of three pelleted diets, exclusively.

	Total OTUs		Unidentified OTUs Removed		Total GS[4]	Unidentified GS[4]
Diet[3]	**J'[1]**	**H'[2]**	**J'[1]**	**H'[2]**		
CNTRL	0.6921 (± 0.01)	4.5751 (±0.09)	0.6369 (± 0.02)	3.3352 (± 0.08)	39,479 (± 7154)	21, 596 (± 3885)
2.0 mm	0.6906 (± 0.01)	4.5580 (± 0.09)	0.5800 (± 0.04)	3.0170 (±0.25)	37,457 (± 9703)	18, 308 (± 4390)
0.2 mm	0.6910 (± 0.02)	4.5658 (± 0.16)	0.5868 (± 0.05)	3.0460 (± 0.27)	41,661 (± 7533)	22, 074 (± 4556)

[1]J', diversity index; measures the normalized numbers of different phyla or genera in samples
[2]H', diversity index; measures how evenly the abundance of phyla or genera are spread in samples
[3]Nutritionally complete, herbivorous tortoise diet, pelleted (Mazuri® 5M21, CNTRL, *n* = 4), and the same tortoise diet with added 2.0 (2.0 mm, *n* = 6) or 0.2 (0.2 mm, *n* = 6) mm length cellulose fiber
[4]GS, gene sequence

Analysis (ANOSIM) of bacterial phyla revealed a difference (*P* = 0.008) by diet. Pairwise comparisons revealed that bacterial phyla were not different between the 2.0 and 0.2 mm diets (*P* = 0.422), but were different for each cellulose-added diet compared to the CNTRL diet (*P* = 0.01 and *P* = 0.014, respectively). Analysis (ANOSIM) of the bacterial genera also revealed a difference (*P* = 0.002) by diet. Similar to phyla, pairwise comparisons revealed that bacterial genera were not different between the 2.0 and 0.2 mm diets (*P* = 0.517), but were different for each cellulose-added diet compared to the CNTRL diet (*P* = 0.01 and *P* = 0.005, respectively).

Diversity indices (J' and H') generated at the level of phyla were not different based on diet (*P* = 0.924, *P* = 0.797, respectively), but were different at the level of genera based on diet (*P* = 0.040, *P* = 0.020, respectively) (Table 14). Tukey's pairwise comparisons at the level of genera revealed that J' was significantly different between tortoises fed the 2.0 mm diet and the CNTRL diet, while those fed the 0.2 mm diet were not significantly

different from either other diet. Tukey's pairwise comparisons (genera-level) for H' revealed that tortoises fed the 2.0 mm and 0.2 mm diets were each significantly different from the CNTRL diet, but not significantly different from one another. Average contribution (%) of bacterial phyla and genera are presented by diet (Table 15).

Table 14. Diversity indices (± standard deviation) of fecal bacterial phyla and genera for leopard tortoises (*Stigmochelys pardalis*) fed one of three pelleted diets, exclusively.

	Phyla		**Genera**	
Diet[3]	**J'[1]**	**H'[2]**	**J'[1]**	**H'[2]**
CNTRL	0.5124 (± 0.01)	1.3523 (± 0.04)	0.6543[a] (± 0.02)	3.0873[a] (± 0.11)
2.0 mm	0.5046 (± 0.04)	1.3193 (± 0.11)	0.6129[b] (± 0.03)	2.8577[b] (± 0.13)
0.2 mm	0.5091 (± 0.03)	1.3435 (± 0.07)	0.6197[a,b] (± 0.02)	2.8838[b] (± 0.10)

[1]J', diversity index; measures the normalized numbers of different phyla or genera in samples

[2]H', diversity index; measures how evenly the abundance of phyla or genera are spread in samples

[3]Nutritionally complete, herbivorous tortoise diet, pelleted (Mazuri® 5M21, CNTRL, n = 4), and the same tortoise diet with added 2.0 (2.0 mm, n = 6) or 0.2 (0.2 mm, n = 6) mm length cellulose fiber

Different letters indicate significant differences ($P < 0.05$)

Table 15. Fecal bacterial phyla and genera mean contributions (≥ 4% and 5%, respectively) of leopard tortoises fed one of three nutritionally complete, pelleted herbivorous tortoise diets, exclusively.

Diet	Phyla	Contribution, %	SD[4]	Genera	Contribution, %	SD[4]
CNTRL[1]	Bacteroidetes	47.98	7.65	*Clostridium*	20.19	2.91
	Firmicutes	34.76	7.49	*Alkaliflexus*	13.67	3.91
	Spirochaetes	4.61	1.11	*Bacteroides*	9.00	3.73
	Proteobacteria	4.56	1.06	*Solitalea*	6.65	5.39
				Ruminococcus	4.95	3.03
2.0 mm[2]	Bacteroidetes	43.34	6.74	*Clostridium*	19.92	2.63
	Firmicutes	41.63	3.88	*Cytophaga*	18.79	4.14
	Spirochaetes	7.22	3.97	*Bacteroides*	10.97	4.84
				Treponema	7.73	3.88
				Ruminococcus	7.34	2.74
				Solitalea	5.50	2.06
0.2 mm[3]	Firmicutes	44.06	5.47	*Clostridium*	24.19	4.20
	Bacteroidetes	42.07	5.85	*Bacteroides*	13.87	2.00
	Spirochaetes	4.33	1.27	*Cytophaga*	13.33	2.05
				Solitalea	6.39	2.08
				Ruminococcus	6.22	0.94
				Treponema	5.07	1.21

[1]Nutritionally complete, herbivorous tortoise diet, pelleted (Mazuri® 5M21; $n = 4$)
[2]Nutrtionally complete, pelleted herbivorous tortoise diet (Mazuri® 5M21) with added 2.0 mm length cellulose fiber ($n = 6$)
[3]Nutrtionally complete, pelleted herbivorous tortoise diet (Mazuri® 5M21) with added 0.2 mm length cellulose fiber ($n = 6$)
[4]SD, standard deviation

Average similarities (SIMPER) among bacterial phyla by diet were 89.1% (CNTRL), 83.9% (2.0 mm), and 86.2% (0.2 mm). Analysis of phyla similarity by diet revealed three distinct groups with > 92% similarity (Fig. 6). All but one individual fed the 0.2 mm diet formed one distinct group, along with three individuals fed the 2.0 mm diet. The other three individuals fed the 2.0 mm diet formed one distinct group themselves. All individuals fed the CNTRL diet formed the third distinct group, along with one individual fed the 0.2 mm diet.

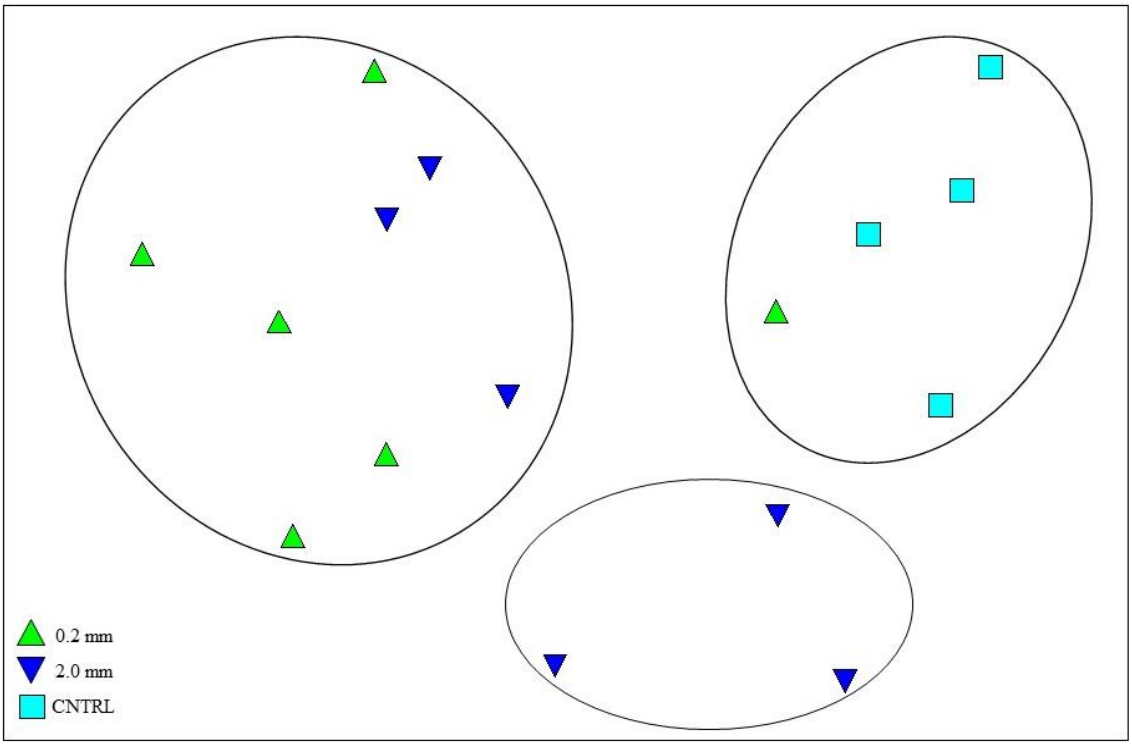

Figure 6. Fecal bacterial phylum similarity (> 92%) of leopard tortoises fed a nutritionally complete, pelleted herbivorous tortoise diet (CNTRL, *n* = 4), and the same tortoise diet with added 2.0 mm (2.0 mm, *n* = 6) or 0.2 mm (0.2 mm, *n* = 6) length cellulose fiber.

Average similarities (SIMPER) among bacterial genera by diet were 78.3% (CNTRL), 75.6 % (2.0 mm), and 77.2% (0.2 mm). Analysis of genera similarity revealed two distinct groups with > 80% similarity (Fig. 7). One group was formed with all but one individual fed the 0.2 mm diet, along with four individuals fed the 2.0 mm diet. The other

group was formed with all individuals fed the CNTRL diet, one individual fed the 0.2 mm diet, and two individuals fed the 2.0 mm diet.

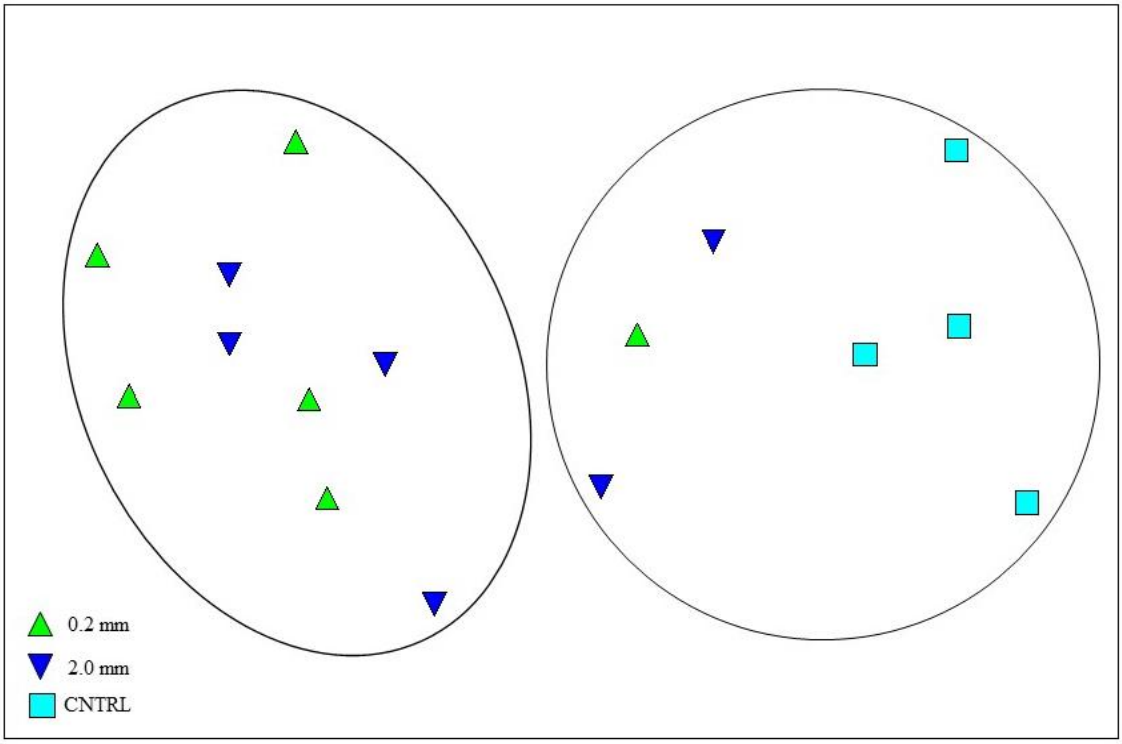

Figure 7. Fecal bacterial genus similarity (> 80%) of leopard tortoises fed a nutritionally complete, pelleted herbivorous tortoise diet (CNTRL, *n* = 4), and the same tortoise diet with added 2.0 mm (2.0 mm, *n* = 6) or 0.2 mm (0.2 mm, *n* = 6) length cellulose fiber.

6.4 Discussion

Analysis of diversity indices indicate similar genera diversity (J') and spread (H') among tortoises fed the cellulose-added diets (2.0 and 0.2 mm). However, diversity was greater for tortoises fed the CNTRL diet compared to the 2.0 mm diet, and spread was greater for tortoises fed the CNTRL diet compared to both the cellulose-added diets (2.0 and 0.2 mm). Since cellulose, as a single ingredient, composed 16%, by weight, of the 2.0 and 0.2 mm diets, perhaps that changed the need for such diversity and spread of hindgut bacteria in individuals fed the cellulose-added diets. All individuals fed the CNTRL diet were grouped together based on both bacterial phyla (Fig. 6) and genera (Fig. 7), suggesting

consistency among these individuals. However, not all individuals fed either the 0.2 or 2.0 mm diet were grouped together. One individual fed the 0.2 mm diet was consistently grouped with all individuals fed the CNTRL diet, suggesting the fecal bacterial profile was more similar to individuals fed the CNTRL diet than to the other individuals fed the 0.2 mm diet. The other five individuals fed the 0.2 mm diet were consistently grouped together, suggesting similarity among their fecal bacterial profiles. For both bacterial phyla and genera, individuals fed the 2.0 mm diet were split. In terms of bacterial phyla, three individuals were grouped with five individuals fed the 0.2 mm diet, while the other three formed a distinct group themselves, suggesting the least consistency among the fecal bacterial profiles of individuals fed the 2.0 mm diet. In terms of bacterial genera, individuals fed the cellulose-added diets were more similar to one another (grouped together more) than they were with the individuals fed the CNTRL diet, and therefore, were more similar at the level of genera than at the level of phyla. Average contribution (%) of Bacteroidetes and Firmicutes found in *S. pardalis* are similar to those reported in free-ranging gopher tortoises (*Gopherus polyphemus*) (Gaillard, 2014; Yuan etal., 2015) (Fig. 8), compared to other hindgut-fermenting reptiles (Table 12).

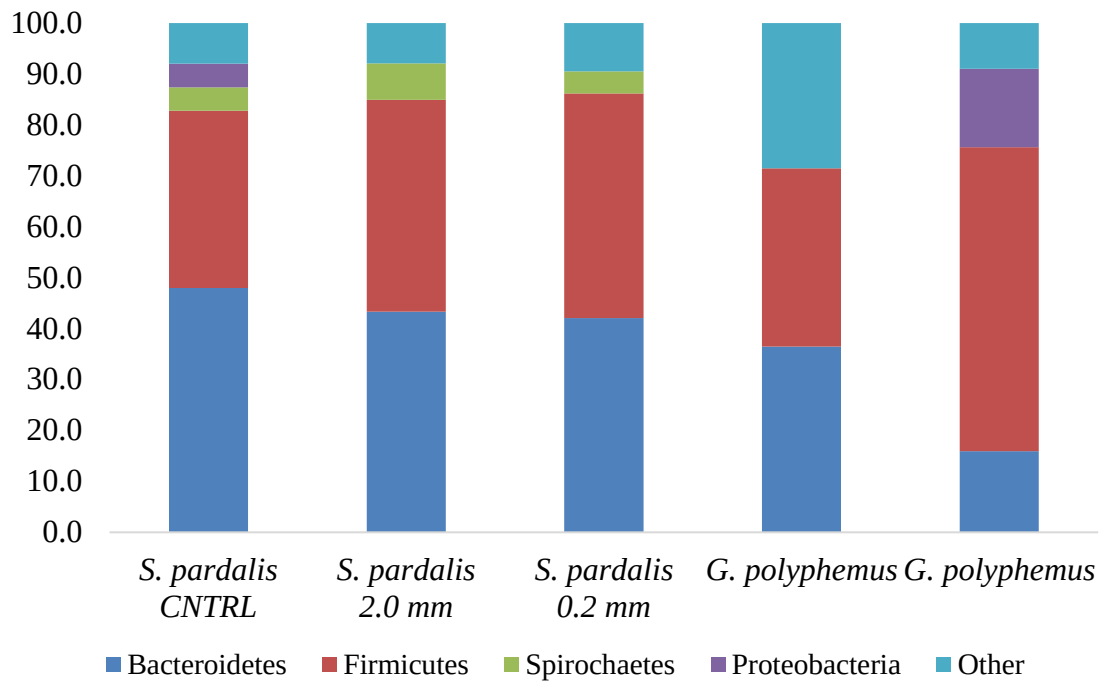

Figure 8. Fecal bacterial phyla of leopard tortoises fed a pelleted herbivorous tortoise diet (CNTRL, *n* = 4), the same diet with added 2.0 mm (2.0 mm, *n* = 6) or 0.2 mm length cellulose fiber (0.2 mm, *n* = 6), and free-ranging gopher tortoises (Gaillard, 2014; Yuan et al., 2015).

This may be due, in part, to dietary differences of the reported species. It has been reported that free-ranging *S. pardalis*, categorized as a 'dome-shelled' tortoise with a short-reaching neck, tends to choose shorter, rather than taller, grasses and other plant parts close to the ground due to their stature (Kabigumila, 2001a). A similar approach to food items may be assumed for *G. polyphemus* due to similar morphology. However, *G. nigra* is categorized as a 'saddleback' tortoise of much greater stature, having the ability to stretch its neck out and up, allowing it to reach taller grasses and plant parts higher up on the stalk (Kabigumila, 2001a). *Amblyrhynchus cristatus*, *Conolophus subcristatus*, *C. pallidus*, and *Iguana iguana* have the ability to reach food items from the ground up into the trees.

With limited reach, and therefore assumedly limited choice in the plant parts for ingestion compared to *G. nigra*, free-ranging *S. pardalis* may be unable to avoid plant parts

that potentially contain toxic substances. Kabigumila (2001a) identified 9 genera (of which 4 were identified to species level) of plants ingested by free-ranging *S. pardalis* that contain toxic substances, including cotyledontoxin, cucurbitacin, cyanin, hepatoxin, oxalic acid, potassium oxalate, and sapogenin. Tortoises with less reach may be able to handle these toxic substances due to the higher concentration of Bacteroidetes, as Bacteroidetes has been identified as possessing pump systems that can rid the bacteria of toxins (Wexler, 2007). While the benefits of possessing gastrointestinal bacteria capable of processing toxins for captive species is unknown, it has been suggested that it allows free-ranging tortoises to avoid competition for grazing material with herbivorous mammalian vertebrates (Lagarde et al., 2003). Of the 24 identified plants ingested by free-ranging *Testudo horsfieldii* (*n* = 7), most were reported to consist of toxins, some known to be harmful to mammalian herbivores, including terpenoids and ranunculin (Lagarde et al., 2003).

At the genus level, our *S. pardalis* colony does not appear similar to any other genera profiles identified in hindgut-fermenting reptiles. Clostridia was identified as approximately 50% of the represented Firmicutes in all free-ranging iguana species and the Aldabra tortoise (Hong et al., 2011), and 97% of the represented Firmicutes in free-ranging gopher tortoises (Yuan et al., 2015). In our captive colony of *S. pardalis*, Clostridia accounted for approximately 20% of represented Firmicutes across all diets. This is surprising, since Clostridia is predominantly cellulolytic. Additionally, Bacteroides, another highly cellulolytic group, was present at less than 15% of represented Bacteroidetes in our *S. pardalis* colony, across all diets. Although the 2.0 and 0.2 mm diets had added cellulose, the diets overall included a high concentration (%) of cell contents compared to cell wall (Table 16). Since the cell contents are easily digested by the host, and do not

require microbial digestion or fermentation, perhaps a lower concentration of cellulolytic bacteria is sufficient. The native diet of related red foot (*Geochelone carbonaria*) and yellow foot (*G. denticulata*) tortoises has been reported to contain 55% cell wall (Moskovits and Bjorndal, 1990) – 23% greater than the highest cell wall concentration in our test diets (Table 16).

Table 16. Concentration (%) of dry matter (DM) cell contents and cell walls of three pelleted diets fed to leopard tortoises (*Stigmochelys pardalis*).

Diet[1]	**Cell Contents, %**[2]	**Cell Wall, %**[3]
CNTRL (*n* = 4)	78.9	21.1
2.0 mm (*n* = 6)	67.7	32.3
0.2 mm (*n* = 6)	73.9	26.1

[1]Nutritionally complete, pelleted herbivorous tortoise diet (Mazuri 5M21®; CNTRL); pelleted tortoise diet with added 2.0 (2.0 mm) or 0.2 (0.2 mm) mm length cellulose fiber
[2][100% DM – (%aNDF - %ash)]
[3]%aNDF - %ash

Along with a difference in diversity, a difference in organisms was also present. Of the major phyla and genera identified from our *S. pardalis* colony (Table 15), one phyla (> 4%) and multiple genera (> 5%) stood out, as they were not reported for other herbivorous, hindgut-fermenting reptiles (Table 12). The phylum Spirochaetes was identified in the microbial profiles of all three diets. Additionally, the genus *Alkaliflexus* was identified in the bacterial profile of the CNTRL diet, *Cytophaga* was identified in the 2.0 and 0.2 mm profiles, *Solitalea* was identified in the CNTRL and 0.2 mm profiles, and *Treponema* was identified in the 2.0 mm profile (Table 14). Dramatically-changed bacterial genera abundance (%) among diets are reported (Table 17).

Table 17. Dramatically-changed bacterial genera abundance (%) of leopard tortoises (*Stigmochelys pardalis*) fed one of three pelleted diets, exclusively.

	Average Abundance (%)		
Genera	**CNTRL[1]**	**2.0 mm[1]**	**0.2 mm[1]**
Cytophaga	3.87	16.40	11.91
Alkaliflexus	11.82	3.90	5.03
Ruminiclostridium	0.96	3.82	4.59
Elusimicrobium	3.71	0.99	0.40
Eubacterium	0.07	1.25	1.03

[1]CNTRL: nutritionally complete, pelleted herbivorous tortoise diet (n = 4); 2.0 mm: tortoise diet with added 2.0 mm length cellulose fiber (n = 6); 0.2 mm: tortoise diet with added 0.2 mm length cellulose fiber (n = 6)

Spirochaetes is a bacterial phylum that includes one class (Spirochaetia) and one order (Spirochaetales) housing four families (Parte, 2010). The action of members of this phylum are still unclear, but it has been hypothesized that they form acetate, ethanol, and lactate from glucose metabolism (Lee et al., 2013, 2015). A recent study demonstrated the ability of Spirochaetes to oxidize acetate (Lee et al., 2015). Acetate oxidation has been recognized as a methanogenic pathway that occurs when high temperatures are present, or there are other inhibitory conditions for normal methane production (Karakashev et al., 2006). Spirochaetes (i.e. *Brachyspira* (*Serpulina*) *pilosicoli*) has been implicated in the presence of swine dysentery (Fellström and Gunnarsson, 1995; Hampson et al., 2000), and poultry intestinal infections resulting in slowed growth and poor egg production (Dwars et al., 1989; McLaren et al., 1997). The same negative effects of Spirochaetes has not yet been reported for reptiles.

Alkaliflexus is a genus in the family Marinilabiliaceae (order Bacteroidales, class Bacteroides, phylum Bacteroidetes) (Parte, 2010). *Alkaliflexus imshenetskii* has been identified as a cellobiose-degrader (Detkova and Kevbrin, 2009). As previously described, cellobiose is the functional unit of cellulose, comprised of two glucose monosaccharides. With the ability to utilize both β-glucosidase and phosphorylase, *A. imshenetskii* produces

primarily succinate and acetate, and small amounts of formate (Detkova and Kevbrin, 2009). Acetate is the primary short chain fatty acid produced in hindgut-fermentation of the domestic horse (NRC, 2007).

Cytophaga is a genus in the family Cytophagaceae (order Cytophagales, class Cytophagia, phylum Bacteroidetes) (Parte, 2010). *Cytophaga hutchinsonii* has been identified as cellulolytic, hydrolyzing cellulose into cellobiose (Zhu et al., 2013). While both endogluconases and glucosidases have been identified in *C. hutchinsonii*, cellobiohydrolases have not, suggesting that this organism may have other, yet-unidentified, methods for cellulose digestion (Xie et al., 2007).

Solitalea is in the family Sphingobacteriaceae (order Sphingobacteriales, class Sphingobactera, phylum Bacteroidetes (Parte, 2010). Information on the role of *Solitalea* in the gut is still lacking. However, a recent study isolated two genes for α-mannosidase, indicating it has an ability to degrade mannose monosaccharides, and possibly mannoglucans (Liu et al., 2016). Van Soest (1987) defines mannan (a carbohydrate composed of mannose monosaccharides) to be a fermented component of plant material that yields short chain fatty acids (acetate, propionate, and butyrate), reasoning for the presence of *Solitalea* in the hindgut of our *S. pardalis* colony.

Treponema is in the family Spirochaetaceae (order Spirochaetales, class Spirochaetia, phylum Spirochaetes) (Parte, 2010). Multiple species of *Treponema* have been identified as causing diseases such as syphilis (*T. pallidum*, Fraser et al., 1998; Liu et al., 2001), periodontal disease (*T. denticola*, Simonson et al., 1988; Seshadri et al., 2004), and dysentery (*T.* h*yodysenteriae*, Kinyon and Harris, 1979; Lysons et al., 1991). Its

presence in the fecal bacteria of our closed colony are surprising. At the time of this research, no animals exhibited any signs or symptoms of disease.

Despite the initial assessment that cellulolytic bacteria appeared to be lacking in the microbial profiles of our captive *S. pardalis* colony, we have demonstrated a possibly greater breadth of cellulolytic organisms in our colony than has been reported in free-ranging herbivorous, hindgut-fermenting reptiles. This may be due to the greater amount of carbohydrate sources encountered in captive diets compared to native diets of free-ranging individuals. Components of our test diets, in order per the manufacturer, included ground soybean hulls, ground corn, (fiber source), dehulled soybean meal, ground oats, wheat middlings, cane molasses, dehydrated alfalfa meal, wheat germ, dicalcium phosphate, soybean oil, brewers dried yeast, calcium carbonate, salt, dl-methionine, choline chloride, pyridoxine hydrochloride, d-alpha tocopherol acetate, biotin, cholecalciferol, menadione sodium bisulfite complex, calcium pantothenate, vitamin A acetate, folic acid, riboflavin, mixed tocopherols, rosemary extract, nicotinic acid, vitamin B_{12} supplement, thiamine mononitrate, citric acid, l-lysine, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, sodium selenite, and cobalt carbonate. Only the 2.0 and 0.2 mm diets included the “fiber source” (cellulose).

7. CONCLUSIONS

The preceding studies not only demonstrate effects of insoluble fiber on the gastrointestinal tract of a hindgut-fermenting vertebrate, but demonstrate effects across levels (i.e., particle size, digestibility, microbial communities). Evidence includes 1) differing changes in particle size (from diet to fecal state) across diets, with similar fecal particle size distributions across diets, 2) higher aNDF and sADF digestibility for tortoises fed the cellulose-added diets, and 3) a shift in the proportions of microbes, with cellulose-degrading microbes in higher proportions in feces from tortoises fed the cellulose-added diets. The quantified change in particle size of the cellulose-added diets may have resulted in higher aNDF and sADF digestibility, which in turn, may be due to shifts in microbial populations of cellulose-degrading bacteria, resulting from higher aNDF and sADF concentrations of the cellulose-added diets. Higher aNDF and sADF digestibility may result in greater concentrations of short-chain fatty acids, supplying energy to the animal host from an otherwise unusable source.

Based on the definition for physically effective fiber (peNDF) for hindgut-fermenting vertebrates that was used for these studies, it is difficult to conclude whether the tested fiber particle lengths (2.0 and 0.2 mmm) acted as peNDF. These results suggest the definition of peNDF for hindgut-fermenting vertebrates should be revised to include additional components. A possible revision may be: physically effective neutral detergent fiber is the combined influence of fiber particle length and NDF concentration that promotes slower digesta movement in the hindgut, maintaining a healthy hindgut environment for microbial communities and allowing more time for microbial fermentation of structural carbohydrates.

We suggest that future studies consider using fiber lengths greater than 2.0 mm, and isolating these fiber lengths from other dietary components (i.e., using > 2.0 mm Bermuda grass (*Cynodon dactylon*) hay pieces instead of a pelleted or extruded diet). The isolation of the fiber particles would allow for a fecal particle size distribution representative solely of one fiber particle length. Since particle interactions occur along the gastrointestinal tract, singling out particle sizes to understand their individual role in the gut may be beneficial to understanding their interaction with other particles. This would also allow for comparison of fecal microbial communities, to further investigate the influence of particle size on the hindgut environment.

WORKS CITED

Agazzi, A., M. Ferroni, A. Fanelli, S. Maroccolo, G. Invernizzi, V. Dell'Orto, and G. Savoini. 2011. Evaluation of the effects of live yeast supplementation on apparent digestibility of high-fiber diet in mature horses using the acid insoluble ash modified method. J. Equine Vet. Sci. 31: 13-18. doi: 10.1016/j.jevs.2010.11.012

Alwash, A. H., and P. C. Thomas. 1971. The effect of the physical form of the diet and the level feeding on the digestion of dried grass by sheep. J. Sci. Food Agr. 22: 611-615.

Amerah, A. M., R. G. Lentle, and V. Ravindran. 2007. Influence of feed form on gizzard morphology and particle size spectra of duodenal digesta in broiler chicks. J. Poult. Sci. 44: 175-181. doi: 10.2141/jpsa.44.175

Amor, K. B, and E. E. Vaughan. 2006. The gastrointestinal microbiota of farm animals. In: Ouwehand, A. C., and E. E. Vaughan, editors. Gastrointestinal microbiology. Taylor and Francis Group, LLC. New York, NY. Pp. 1-24.

Andreesen, J. R., H. Bahl, and G. Gottschalk. Introduction to the physiology and biochemistry of the genus *Clostridium*. In: Minton, N. P., and D. J. Clarkes, editors. Clostridia. Biotechnology Handbooks. Vol 3. Plenum Press, New York and London. Pp. 27-62.

Ankom. 2014. Acid detergent fiber in feeds – filter bag technique (A200 and A2001). Method 5. Ankom Technology.

Ankom, 2015. Neutral detergent fiber in feeds – filter bag technique (A200 and A2001). Method 13. Ankom Technology.

Arthur, K. E., S. Kelez, T. Larsen, C. A. Choy, and B. N. Popp. 2014. Tracing the biosynthetic source of essential amino acids in marine turtles using δ13C fingerprints. Ecology. 95: 1285-1293. doi: 10.1890/13-0263.1

Atlas, R. M., and R. Bartha. 1998. Microbial Ecology: Fundamentals and applications. Benjamin/Cummings, The University of California.

Barboza, P. S. 1995. Digesta passage and functional anatomy of the digestive tract in the desert tortoise (*Xerobates agassizii*). J. Comp. Physiol. B. 165: 193-202.

Bayer, E. A., L. J. Shimon, Y. Shoham, and R. Lamed. 1998. Cellulosomes – structure and Uultrastructure. J. Struct. Biol. 124: 221-234.

Bennett, D. C., H. M. Tun, J. E. Kim, F. C. Leung, and K. M. Cheng. 2013. Characterization of cecal microbiota of the emu (*Dromaius novaehollandiae*). Vet. Microbiol. 16: 304-310. doi: 10.1016/j.vetmic.2013.05.018

Bjorndal, K. A. 1989. Flexibility of digestive responses in two generalist herbivores, the tortoises *Geochelone carbonaria* and *Geochelone denticulata*. Oecologia. 78(3): 317-321.

Bjorndal, K. A., A. B. Bolten, and J. E. Moore. 1990. Digestive fermentation in herbivores: Effect of food particle size. Physiol. Zool. 63(4): 710-721.

Bjorndal, K. A. 1991. Digestive fermentation in green turtles, *Chelonia mydas*, feeding on algae. Bull. Marine Sci. 48: 166-171.

Bjorndal, K. A. 1997. Fermentation in reptiles and amphibians. In: Mackie, R. I. and B. A. White, editors, Gastrointestinal Microbiology. Springer US. p. 199-225.

Bouchard, S. S., and K. A. Bjorndal. 2005. Microbial fermentation in juvenile and adult pond slider turtles, *Trachemys scripta*. J. Herpetol. 39(2): 321-324.

Boycott, R. C., and O. Bourquin. 1988. The South African Tortoise Book. Southern Book Publishers, Johannesburg. Pp. 86-90.

Boyer, T. H., and D. M. Boyer. 2006. Turtles, tortoises, and terrapins. In: Mader. D. R. Reptile medicine and surgery. 2nd ed. Saunders Elsevier. St. Louis, MO. Pp. 82.

Bush E. R., S. E. Baker, and D. W. MacDonald. 2014. Global trade in exotic pets 2006–2012. Conserv. Biol. 28(3): 663-676. doi: 10.1111/cobi.12240

Carere, C. R., R. Sparling, N. Cicek, and D. B. Levin. 2008. Third generation biofuels via direct cellulose fermentation. Int. J. Mol. Sci. 9(7): 1342-1360. doi: 10.3390/ijms9071342

Carmalt, J. L., N. F. Cymbaluk, and H. G. G. Townsend. 2005. Effect of premolar and molar occlusal angle on feed digestibility, water balance, and fecal particle size in horses. JAVMA. 227(1): 110-113. doi: 10.2460/javma.2005.227.110

Carmalt, J. L., and A. L. Allen. 2006. Effect of rostrocaudal mobility of the mandible on feed digestibility and fecal particle size in horses. JAVMA. 229(8): 1275-1278. doi: 10.2460/javma.229.8.1275

Carmalt, J. L., and A. L. Allen. 2008. The relationship between cheek tooth occlusal morphology, apparent digestibility, and ingesta particle size reduction in horses. JAVMA. 233(3): 452-455. doi: 10.2460/javma.233.3.452

CITES. 2014a. United Nations Environment Programme. Appendices I, II, and III. http://www.cites.org (Accessed 15 October 2015.)

CITES. 2014b. Commission implementing regulation (EU) No 888/2014 of 14 August 2014 prohibiting the introduction into the Union of specimens of certain species of

wild flora and fauna. Official Journal of the European Union. I. 243/21. http://www.cites.org (Accessed 15 October 2015.)

Clauss, M., M. Lechner-Doll, and W. J. Streich. 2002. Faecal particle size distribution in captive wild ruminants: an approach to the browser/grazer dichotomy from the other end. Oecologia. 131: 343-349. doi: 10.1007/s00442-002-0894-8

Clauss, M., C. Nunn, J. Fritz, and J. Hummel. 2009. Evidence for a tradeoff between retention time and chewing efficiency in large mammalian herbivores. Comp. Bioch. Physiol. Part A. Mol. Integr. Physiol. 154: 376-382. doi: 10.1016/j.cbpa.2009.07.016

Clauss, M., P. Steuer, K. Erlinghagen-Lückerath, J. Kaandorp, J. Fritz, K-H. Südekum, and J. Hummel. 2015. Faecal particle size: Digestive physiology meets herbivore diversity. Comp. Bioch. Physiol. Part A. Mol. Integr. Physiol. 179: 182-191. doi: 10.1016/j.cbpa.2014.10.006

Danson, M. J., H. J. Lamble, and D. W. Hough. 2007. Central metabolism. And Methanogenesis. In: Cavicchioli, R., editor. Archaea. Molecular and cellular SM Press, Washington, DC. Pp. 260-314.

Davis, S. 1979. Husbandry and breeding of the red-footed tortoise (*Geochelone carbonaria*) at the National Zoological Park, Washington. Int. Zoo Yearb. 19(1): 50-53.

Detkova, E. N., and V. V. Kevbrin. 2009. Cellobiose catabolism in the haloalkaliphilic hydrolytic bacterium *Alkaliflexus imshenetskii*. Microbiology. 78(3): 267-272.

Diaz-Figueroa, O., and M. A. Mitchell. 2006. Gastrointestinal anatomy and physiology. In: Mader, D. R. Reptile medicine and surgery. 2nd ed. Saunders Elsevier. St. Louis, MO. Pp. 148.

Dodd, D., R. I. Mackie, and I. K. O. Cann. 2011. Xylan degradation, a metabolic property shared by rumen and human colonic Bacteroidetes. Mol. Microbiol. 79(2): 292-304. doi: 10.1111/j.1365-2958.2010.07473.x.

Doi, R. H., and A. Kosugi. 2004. Cellulosomes: Plant-cell-wall-degrading enzyme complexes. Nat. Rev. Microbiol. 2: 541-551. doi:10.1038/nrmicro925

Donoghue, S., and S. McKeown. 1999. Nutrition of captive reptiles. Vet. Clin. North Am. Exot. Anim. Pract. 2(1): 69-91.

Doyle, P. T., T. Casson, L. Cransberg, and J. B. Rowe. 1994. Faecal output of grazing sheep measured by total collection or using chromium sesquioxide. Small Rumin. Res. 13: 231.236.

Dwars, R. M., H. F. Smit, F. G. Davelaar, and W. Van T Veer. 1989. Incidence of spirochaetal infections in cases of intestinal disorder in chickens. Avian Path. 18(4): 591-595. doi: 10.1080/03079458908418634

Edwards, M. E. 1997. Leaf-eating primates: Nutrition and dietary husbandry. Nutritional Advisory Group Handbook. Association of Zoos and Aquariums.

Fellström, C., and A. Gunnarsson. 1995. Phenotypical characterization of intestinal spirochaetes isolated from pigs. Res. Vet. Med. 59(1): 1-4. doi: 10.1016/0034-5288(95)90021-7

Fledelius, B., G. W. Jorgensen, H. E. Jensen, and L. Brimer. 2005. Influence of the calcium content of the diet offered to leopard tortoises (*Geochelone pardalis*). Vet. Rec. 156: 831-835.

Franz, R., J. Hummel, D. W. H. Muller, M. Bauert, J. M. Hatt, and M. Clauss. 2011. Herbivorous reptiles and body mass: Effects on food intake, digesta retention, digestibility and gut capacity, and a comparison with mammals. Comp. Biochem. Physiol. Part A. Mol. Integr. Physiol. 158: 94-101. doi: 10.1016/j.cbpa.2010.09.007

Fraser, C. M., S. J. Norris, G. M. Weinstock, O. White, G. G. Sutton, R. Dodson, M Gwinn, E. K. Hickey, R. Clayton, K. A. Ketchum, E. Sodergren, J. M. Hardham, M. P. McLeod, S. Salzberg, J. Peterson, H. Khalak, D. Richardson, J. K. Howell, M. Chidambaram, T. Utterback, L. McDonald, P. Artiach, C. Bowman, M. D. Cotton, C. Fujii, S. Garland, B. Hatch, K. Horst, K. Roberts, M. Sandusky, J. Weidman, H. O. Smith, and J. C. Venter. 1998. Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. Science. 281(5375): 375-388. doi: 10.1126/science.281.5375.375

Fritz, C., and P. Havaš. 2007. Checklist of chelonians of the world. Vertebr. Zool. 57: 149-368.

Fritz, J., J. Hummel, E. Kienzle, C. Arnold, C. Nunn, and M. Clauss. 2009. Comparative chewing efficiency in mammalian herbivores. Oikos. 118: 1623-1632. doi: 10.1111/j.1600-0706.2009.17807.x

Fritz, J., J. Hummel, E. Kienzle, W. Jurgen Streich, and M. Clauss. 2010a. To chew or not to chew: Fecal particle size in herbivorous reptiles and mammals. J. Exp. Zool. 313A: 579-586. doi: 10.1002/jez.629

Fritz, U., S. R. Daniels, M. D. Hofmeyr, J. Gonzalez, and C. L. Barrio-Amoros, et al. 2010b. Mitochondrial phylogeography and subspecies of the wide-ranging sub-Saharan leopard tortoise *Stigmochelys pardalis* (Testudines: Testudinidae) – a case study for the pitfalls of pseudogenes and GenBank sequences. J. Zool. Syst. Evol. Res. 48(4): 348-359. doi: 10.1111/j.1439-0469.2010.00565.x

Fritz, J., W. Jurgen Streich, A. Schwarm, and M. Clauss. 2012. Condensing results of wet sieving analyses into a single data: A comparison of methods for particle size description. J. Anim. Physiol. Anim. Nutr. (Berl) 96: 783-797. doi: 10.1111/j.1439-0396.2011.01183.x

Gaillard, D. L. 2014. Population genetics and microbial communitites of the gopher tortoise (*Gopherus polyphemus*). PhD Dissertation, University of Southern Mississippi. Hattiesburg, MS.

Galperin, M. Y. 2013. Genome diversity of spore-forming *Firmicutes*. Microbial spectrum. 1(2): TBS-0015-2012. doi: 10.1128/microbiolspectrum.

Grobler, J. H. 1982. The leopard tortoise in the Mountain Zebra National Park. Koedoe. 25: 49-53.

Guard, C. L. 1980. The reptilian digestive system: general characteristics. In: K. Schmidt-Nielsen, L. Bolis, and C. R. Taylor, editors, Comparative Physiology: Primitive Mammals. Cambridge University Press, New York. Pp. 43-51.

Hailey, A., and I. M. Coulson. 1996. Differential scaling of home range area to daily movement distance in two African tortoises. C. J. Zool. 74: 97-102.

Hailey, A. 1997. Digestive efficiency and gut morphology of omnivorous and herbivorous African tortoises. Can. J. Zool. 75: 787-794.

Hamilton, J., and M. Coe. 1982. Feeding, digestion and assimilation of a population of giant tortoises (*Geochelone gigantea* (Schweigger)) on Aldabra atoll. J. Arid Environ. 5: 127-144.

Hampson, D. J., I. D. Robertson, T. La, S. L. Oxberry, and D. W. Pethick. 2000. Influences of diet and accination on colonization of pigs by the intestinal spirochaete *Brachyspira* (*Serpulina*) *pilosicoli*. Vet. Microbiol. 73(1): 75-84. doi: 10.1016/S0378-1135(99)00200-X

Hatt, J., M. Clauss, R. Gisler, A. Liesegang, M. Wanner. 2005. Fiber digestibility in juvenile Galapagos tortoises (Geochelone nigra) and implications for the development of captive animals. Zoo Biol. 24: 185-191. doi: 10.1002/zoo.20039

Heip, C. H. R., P. M. J. Herman, and K. Soetaert. 1998. Indices of diversity and evenness. Océanis. 24(4): 61-87.

Hong, P., E. Wheeler, I. K. O. Cann, and R. I. Mackie. 2011. Phylogenetic analysis of the fecal microbial community in herbivorous land and marine iguanas of the Galapagos Islands using 16S rRNA-based pyrosequencing. ISME J. 5: 1461-1470. doi: 10.1038/ismej.2011.33

Hooper, L. V., T. Midtvedt, and J. I. Gordon. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Ann. Rev. Nutr. 22: 283-307.

Hummel, J., J. Fritz, E. Kienzle, E. P. Medici, S. Lang, W. Zimmerman, W. J. Streich, and M. Clauss. 2008. Differences in fecal particle size between free-ranging and captive individuals of two browser species. Zoo Biol. 27: 70-77. doi: 10.1002/zoo.20161

Jessop, M. 2009. Identification of tortoises. In Practice. 31: 46-57.

Joshua, Q. I., M. D. Hofmeyr, and B. T. Henen. 2010. Seasonal and site variation in angulate tortoise diet and activity. J. Herpetol. 44(1): 124-134.

Kabigumila, J. 2000. Growth and carapacial colour variation of the leopard tortoise, *Geochelone pardalis babcocki*, in northern Tanzania. Afr. J. Ecol. 38: 217-223.

Kabigumila, J. 2001a. Sighting frequency and food habits of the leopard tortoise, *Geocheone pardalis*, in northern Tanzania. Afr. J. Ecol. 39: 276-285.

Kabigumila, J. 2001b. Occurrence and activity budget of the leopard tortoise Geochelone pardalis, in northern Tanzania. Tanz. J. Sci. 27: 87-99.

Karakashev, D., D. J. Batstone, E. Trably, and I. Angelidaki. 2006. Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of Methanosaetaceae. Appl Environ. Microbiol. 72(7): 5138-5141.

Kinyon, J. M., and D. L. Harris. 1979. *Treponema innocens*, a new species of intestinal bacteria, and emended description of the type strain of *Treponema hyodysenteriae* Harris et al. Int. J. Syst. Bacteriol. 29(2): 102-109.

Klasing, K. 2005. Interplay between diet, microbes, and immune defenses of the gastrointestinal tract. In: J. M. Starck and T. Wang, editors, Physiological and Ecological Adaptations to Feeding in Vertebrates. Science Publishers, New Hampshire. Pp. 255-277.

Kormelink, T. G., E. Koenders, Y. Hagemeijer, L. Overmars, R. J. Siezen, W. M. de Vos, and C. Francke. 2012. Comparative genome analysis of central nitrogen metabolism and its control by GlnR in the class bacilli. BMC Genomics. 13: 191. doi: 10.1186/1471-2164-13-191

Kostic, A.D., M. R. Howitt, and W. S. Garrett. 2013. Exploring host-microbiota interactions in animal models and humans. Genes Dev. 27: 701-718. doi: 10.1101/gad.212522.112

Lagarde, F., X. Bonnet, J. Corbin, B. Henen, K. Nagy, B. Mardonov, and G. Naulleau. 2003. Foraging behaviour and diet of an ectothermic herbivore: *Testudo horsfieldi*. Ecography. 26: 236-242.

Lamed, R., and E. A. Bayer. 1986. Contact and cellulolysis in Clostridium thermocellum via extensive surface organelles. Experientia. 42: 72-73.

Le, M., C. J. Raxworthy, W. P. McCord, and L. Mertz. 2006. A molecular phylogeny of tortoises (Testudines: Testudinidae) based on mitochondrial and nuclear genes. Mol. Phylog. Evol. 40: 517-531. doi: 10.1016/j.ympev.2006.03.003

Lee, S. H., J. H. Park, H. J. Kang, Y. H. Lee, J. J. Lee, and H. D. Park. 2013. Distribution and abundance of Spirochaetes in full-scale anaerobic digesters. Biores. Technol. 145: 25. doi: 10.1016/j.biortech.2013.02.070

Lee, S. H., J. H. Park, S. H. Kim, B. J. Yu, J. J. Yoon, and H. D. Park. 2015. Evidence of syntrophic acetate oxidation by Spirochaetes during anaerobic methane production. Biores. Techonol. 190: 543-549. doi: 10.1016/j.biortech.2015.02.066

Leser, T. D., and L. Mølbak. 2009. Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. Environ. Microbiol. 11(9): 2194-2206. doi: 10.1111/j.1462-2920.2009.01941.x

Ley, R.E., C. A. Lozupone, M. Hamady, R. Knight, and J. I. Gordon. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. Nat. Rev. Microbiol. 6: 776-788. doi: 10.1038/nrmicro1978

Lickel, L.E. 2010. Intake, apparent digestibility, and digesta passage in leopard tortoises (*Geochelone pardalis*) fed a complete, extruded feed. MS Thesis, California Polytechnic State University. San Luis Obispo, CA.

Liu H., B. Rodes, C.-Y. Chen, and B. Steiner. 2001. New tests for syphilis: Rational design of a PCR method for detection of *Treponema pallidum* in clinical specimens using unique regions of the DNA polymerase I gene. J. Clin. Microbiol. 39(5): 1941-1946. doi: 10.1128/JCM.39.5.1941–1946.2001

Liu, F. F., A. Kulinich, Y. M. Du, L. Liu, and J. Voglmeir. 2016. Sequential processing of mannose-containing glycans by two α-mannosidases from *Solitalea canadensis*. Glycoconj. J. 33(2): 159-168. doi: 1007/s10719-016-9651-9

Lysons, R. J., K. A. Kent, A. P. Bland, R. Sellwood, W. F. Robinson, and A. J. Frost. 1991. A cytotoxic haemolysin from Treponema hyodysenteriae – a probable virulence determinant in swine dysentery. J. Med. Microbiol. 34: 97-102.

Marlow, R. W., and K. Tollestrup. 1982. Mining and exploitation of natural mineral deposits by the desert tortoise, *Gopherus agassizii*. Anim. Behav. 30: 475-478.

Matsuda, I., A. Tuuga, C. Hashimoto, H. Bernard, J. Yamagiwa, J. Fritz, K. Tsubokawa, M. Yayota, T. Murai, and M. Clauss. 2014. Faecal particle size in free-ranging primate supports a 'rumination' strategy in the proboscis monkey (*Nasalis larvatus*). Oecologia. 174: 1127-1137. doi: 10.1007/s00442-013-2863-9

Mazuri. 2016. Mazuri® tortoise LS diet. http://www.mazuri.com/product_pdfs/5E5L.pdf (Accessed 20 June 20 2016.)

McBee, R. H., and B. H. McBee. 1982. The hindgut fermentation in the green iguana, *Iguana iguana*. In: Burghardt, G. M., and A. S. Rand, editors. Iguanas of the world:

their behavior, ecology and conservation. Noyes Publications. Park Ridge, NJ. Pp. 77-82.

McLaren, A. J., D. J. Trott, D. E. Swayne, S. L. Oxberry, and D. J. Hampson. 1997. Genetic and phenotypic characterization of intestinal spirochetes colonizing chickens and allocation of known pathogenic isolates to three distinct genetic groups. J. Clin. Microbiol. 35(2): 412-417.

McMaster, M. K., and C. T. Downs. 2006a. Population structure and density of leopard tortoises (*Geochelone pardalis*) on farmland in the Nama-Karoo. J. Herpetol. 40(4): 495-502.

McMaster, M. K., and C. T. Downs. 2006b. Do seasonal and behavioral differences in the use of refuges by the leopard tortoise (*Geochelone pardalis*) favor passive thermoregulation? Herpetologica. 62(1): 37-46.

McMaster, M. K., and C. T. Downs. 2008. Digestive parameters and water turnover of the leopard tortoise. Comp. Biochem. Physiol. Part A. Mol. Integr. Physiol. 151: 114-125. doi: 10.1016/j.cbpa.2008.06.007

McMaster, M. K., and C. T. Downs. 2009. Home range and daily movement of leopard tortoises (*Stigmochelys pardalis*) in the Nama-Karoo, South Africa. J. Herpetol. 43(4): 561-569.

McMaster, M. K., and C. T. Downs. 2013a. Seasonal and daily activity patterns of leopard tortoises (*Stigmochelys pardalis* Bell, 1828) on farmland in the Nama-Karoo, South Africa. Afr. Zool. 48(1): 72-83. doi: 10.3377/004.048.0110

McMaster, M. K., and C. T. Downs. 2013b. Thermoregulation in leopard tortoises in the Nama-Karoo: The importance of behaviour and core body temperatures. J. Therm. Biol. 38: 178-185. doi: 10.1016/j.jtherbio.2013.02.003

Merchen, N. R. 1988. Digestion, absorption and excretion in ruminants. In: Church, D. C., editor. The ruminant animal: Digestive physiology and nutrition. Prentice Hall, Englewood Cliffs, NJ. Pp. 172-201.

Mertens, D. R. 1997. Creating a system for meeting the fiber requirements of dairy cows. J. Dairy Sci. 80: 1463-1481.

Miller, T. L., and M. J. Wolin. 1979. Fermentations by saccharolytic intestinal bacteria. Am. J. Clin. Nutr. 32: 164-172.

Millette, J. B., M. L. Sauther, F. P. Cuozzo, J. L. Ness. 2012. The impact of dental impairment on ring-tailed lemur food processing performance. Am. J. Phys. Anthropol. 148: 238-248. doi: 10.1002/ajpa.21571

Milton, S. J. 1992. Plants eaten and dispersed by adult leopard tortoises *Geochelone pardalis* (Reptilia: Chelonii) in the Southern Karoo. S. Afr. J. Zool. 27: 45-49.

Moskovits, D. K., and K. A. Bjorndal. 1990. Diet and food preferences of the tortoises *Geochelone carbonaria* and *G. denticulata* in northwestern Brazil. Herpetologica. 46(2):207-218.

Nagy, K. A., I. A. Girard, and T. K. Brown. 1999. Energetics of free-ranging mammals, reptiles, and birds. Ann. Rev. Nutr. 19: 247-277.

National Research Council (NRC). 2007. Nutrient requirements of horses. 6th rev ed. National Academy Press, Washington, DC.

Norman, D. B., and D. B. Weishampel. 1985. Ornithopod feeding mechanisms: their bearing on the evolution of herbivory. Am. Soc. Nat. 126(2): 151-164.

Owens, F. N., and A. L. Goetsch. 1988. Ruminal fermentation. In: Church, D. C., editor. The ruminant animal: Digestive physiology and nutrition. Prentice Hall, Englewood Cliffs, NJ. Pp. 145-171.

Parte, A. C. 2010. Bergey's manual of systematic bacteriology. 2nd ed. Springer. New York, NY.

Patterson, R. 1987. Leopard or mountain tortoise. In: Reptiles of Southern Africa. C. Struik Publishers. Cape Town, South Africa. Pp. 18-19.

Poppi, D. P., D. J. Minson, and J. H. Ternouth. 1981. Studies of cattle and sheep eating leaf and stem fractions of grasses. Factors controlling the retention of feed in the reticulo-rumen. Austr. J. Agr. Res. 32(1): 109-121. doi: 10.1071/AR9810109

Porter, K. R. 1972. Herpetology. W.B. Saunders Company, Pennsylvania.

Rall, M., and N. Fairall. 1993. Diets and food preferences of two South African tortoises *Geochelone pardalis* and *Psammobates oculifer*. S. Afr. J. Wildl. Res. 23(3): 63-70.

Ritz, J., M. Clauss, W. Jurgen Streich, and J. M. Hatt. 2012. Variation in growth and potentially associated health status in Hermann's and Spur-Thighed tortoise (*Testudo hermanni and Testudo graeca*). Zoo Biol. 31: 705-717. doi: 10.1002/zoo.20273

Rodrigue, C. B., and N. N. Allen. 1960. The effect of fine grinding of hay on ration digestibility, rate of passage, and fat content in milk. Can. J. Anim. Sci. 40: 23-29.

Sadeghayobi, E., S. Blake, M. Wikelski, J. Gibbs, R. Mackie, and F. Cabrera. 2011. Digesta retention time in the Galapagos tortoise (*Chelonoidis nigra*). Comp. /vioch. Physiol. Part A. Mol. Integr. Physiol. 160: 493-497. doi: 10.1016/j.cbpa.2011.08.008

Salway, J. G. 1999. Metabolism at a glance. 2nd ed. Blackwell Sciences. Oxford, London. Pp. 36.

Schloss, P.D., and S. L. Westcott. 2011. Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis. Appl. Environ. Microbiol. 77(10): 3219-3226. doi: 10.1128/AEM.02810-10.

Schneider, B. H. and W. P. Flatt. 1975. The evaluation of feed through digestibility experiments. The Univeristy of Georgia Press. Athens.

Schwartz, E. M. 2015. Digestibility of two complete pelleted diets by the horse (*Equus caballus*) as a model animal for nondomestic hindgut fermenters. MS Thesis, California Polytechnic State University. San Luis Obispo, CA.

Schwarz, W. H. 2001. The cellulosome and cellulose degradation by anaerobic bacteria. Appl. Microbiol. Biotechnol. 56: 634-649. doi: 10.1007/s002530100710

Schwenk, K., and M. Rubega. 2005. The diversity of vertebrate feeding systems. In: J. M. Starck and T. Wang, editors, Physiological and Ecological Adaptations to Feeding in Vertebrates. Science Publishers, New Hampshire. Pp. 1-41.

Seshadri, R., G. S. A. Meyers, H. Tettellin, J. A. Eisen, J. F. Heidelberg, R. J. Dodson, T. M. Davidsen, R. T. DeBoy, D. E. Fouts, D. H. Haft, J. Selengut, Q. Ren, L. M. Brinkac, R. Madupu, J. Kolonay, S. A. Durkin, S. C. Daugherty, J. Shetty, A.

Schvartzbeyn, E. Gebregeorgis, K. Geer, G. Tsegaye, J. Malek, B. Ayodeji, S. Shatsman, M. P. McLeod, D. Smajs, J. K. Howell, S. Pal, A. Amin, P. Vashisth, T. Z. McNeill, Q. Xiang, E. Sodergren, E. Baca, G. M. Weinstock, S. J. Norris, C. M. Fraser, and I. T. Paulsen. 2004. Comparison of the genome of the oral pathogen Treponema denticola with other spirochete genomes. PNAS. 101(15): 5646-5651. Doi: 10.1073/pnas.0307639101

Sharp, R. J., M. D. Scawen, and T. Atkinson. 1989. Fermentation and downstream processing of *Bacillus*. In: Harwood, C. R., editor. Bacillus. Plenum Press, New York, NY. Pp. 255-292.

Shine, R. 2005. Life-history evolution in reptiles. Ann. Rev. Ecol. Evol. Syst. 36: 23-46. doi: 10.1146/annurev.ecolsys.36.102003.152631

Shirley, R. L. 1986. Nitrogen and energy nutrition of ruminants. Academic Press, Inc. Orlando, FL.

Simang, A., P. L. Cunningham, and B. T. Henen. 2010. Color selection by juvenile leopard tortoises (*Stigmochelys pardalis*) in Namibia. J. Herpetol. 44(2): 327-331.

Simonson, L. G., C. H. Goodman, J. J. Bial, and H. E. Morton. 1988. Quantitative relationship of *Treponema denticola* to severity of periodontal disease. Infec. Immun. 56(4): 726-728.

Skoczylas, R. 1978. Physiology of the digestive tract. In: Gans, C., and K. A. Gans, editors. Biology of the Reptilia. Vol 8. Academic Press. London, United Kingdom. Pp. 589-664.

Spellerberg, I. F. 1982. Biology of Reptiles. Chapman and Hall, New York.

Stevens, C. E., and I. D. Hume. 1998. Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. Physiol. Rev. 78(2): 393-427.

Stevens, C. E., and I. D. Hume. 2004. Comparative Physiology of the Vertebrate Digestive System. 2nd ed. Cambridge University Press, United Kingdom.

Sutton, E. I., J. P. Bowland, and J. F. McCarthy. 1977. Studies with horses comparing 4N-HCl insoluble ash as an index material with total fecal collection in the determination of apparent digestibilities. Can. J. Anim. Sci. 57: 543-549.

Taylor, S. K., S. B. Citino, J. M. Zdziarski, and R. M. Bush. 1996. Radiographic anatomy and barium sulfate transit time of the gastrointestinal tract of the leopard tortoise (*Testudo pardalis*). J. Zoo Wildl. Med. 27(2): 180-186.

Tracy, C. R., L. C. Zimmerman, C. Tracy, K. D. Bradley, and K. Castle. 2006. Rates of food passage in the digestive tract of young desert tortoises: Effects of body size and diet quality. Chelonian Conserv. Biol. 5(2): 269-273.

Troyer, K. 1984. Structure and function of the digestive tract of a herbivorous lizard *Iguana iguana*. Physiol. Zool. 57(1): 1-8.

Ullrey, D. E., S. D. Crissey, and H. F. Hintz. 1997. Elephants: Nutrition and dietary husbandry. Nutritional Advisory Group Handbook. Association of Zoos and Aquariums.

Van Keulen, J., and B. A. Young. 1977. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. J. Anim. Sci. 44(2): 282-287.

Van Soest, P. J. 1987. Nutritional ecology of the ruminant: Ruminant metabolism, nutritional strategies, the cellulolytic fermentation and the chemistry of forages and plant fibers. Cornell University Press, Ithaca NY.

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2nd ed. Cornell University Press, Ithaca, NY.

Wexler, H. M. 2007. Bacteroides: the good, the bad, and the nitty-gritty. Clin. Microbiol. Rev. 20(4): 593-621. doi: 10.1128/CMR.00008-07

White, D. 1995. The physiology and biochemistry of prokaryotes. Oxford University Press. New York, NY.

Wimberger, K., A. J. Armstrong, and M. Penning. 2011. Health checks of rehabilitated leopard tortoise, *Stigmochelys pardalis*, before release into the wild. S. Afr. J. Wildl. Res. 41(2):229-235. doi: 10.3957/056.041.0212

Wrong, O. M., C. J. Edmonds, and V. S. Chadwick. 1981. The Large Intestine: Its Role in Mammalian Nutrition and Homeostasis. Halsted Press, New York.

Xie, G., D. C. Bruce, J. F. Challacombe, O. Chertkov, J. C. Detter, P. Gilna, C. S. Han, S. Lucas, M. Misra, G. L. Myers, P. Richardson, R. Tapia, N. Thayer, L. S. Thompson, T. S. Brettin, B. Henrissat, D. B. Wilson, and M. J. McBride. Genome sequence of the cellulolytic gliding bacterium *Cytophaga hutchinsonii*. Appl. Environ. Microbiol. 73(11): 3536-3546.

Yang, W. Z., and K. A. Beauchemin. 2007. Altering physically effective fiber intake through forage and particle length: Digestion and milk production. J. Dairy Sci. 90: 3410-3421. doi: 10.3168/jds.2006-818

Yokoyama, M. T., and K. A. Johnson. 1988. Microbiology of the rumen and intestine. In: Church, D. C., editor. The ruminant animal: Digestive physiology and nutrition. Prentice Hall, Englewood Cliffs, NJ. Pp. 125-144.

Yuan, M. L., S. H. Dean, A. V. Longo, B. B. Rothermel, T. D. Tuberville, and K. R. Zamudio. 2015. Kinship, inbreeding and fine-scale spatial structure influence gut microbiota in a hindgut-fermenting tortoise. Mol. Ecol. 24: 2521-2536. doi: 10.1111/mec.13169

Zhu, Y., H. Zhou, Y. Bi, W. Zhang, G. Chen, and W. Liu. 2013. Characterization of a family 5 glycoside hydrolase isolated from the outer membrane of cellulolytic *Cytophaga hutchinsonii*. Appl. Microbiol. Biotechnol. 97(9): 3925-3937. doi: 10.1007/s00253-012-4259-x

APPENDICES

A. Physically Effective Fiber Data

Table 18. Dry matter (DM, %) of fecal particle size (mm) of leopard tortoises (*n* = 16) fed one of three nutritionally complete, pelleted herbivorous tortoise diets, exclusively.

		Sieve Size (mm)							
Tortoise	**Diet[1]**	**2.0**	**1.0**	**0.5**	**0.25**	**0.125**	**0.063**	**<0.063**	**S[2]**
307100	CNTRL	0.05	0.29	11.38	18.69	11.04	5.40	0.01	53.14
307107	CNTRL	0.15	0.52	10.53	21.46	13.19	5.54	0.02	48.51
307110	CNTRL	-0.04	0.15	8.45	18.34	10.73	5.59	0.00	56.78
307111	CNTRL	-0.04	0.12	9.73	19.90	12.22	6.90	0.01	51.16
307094	0.2 mm	0.10	0.07	6.24	15.11	7.94	4.37	-0.01	66.17
307097	0.2 mm	0.50	0.34	8.43	15.10	10.08	4.80	0.07	60.67
307098	0.2 mm	-0.04	0.25	8.19	18.65	10.93	5.50	0.00	56.51
307102	0.2 mm	0.16	0.69	7.69	12.83	8.82	5.66	0.02	64.14
307104	0.2 mm	0.04	0.11	8.11	16.89	8.74	4.38	0.01	61.72
307112	0.2 mm	0.05	0.25	7.57	16.29	10.71	4.80	0.02	60.33
3071010	2.0 mm	0.48	0.29	8.35	14.36	9.53	5.27	0.04	61.59
307103	2.0 mm	0.95	0.71	7.71	14.48	9.21	4.73	0.05	62.26
307106	2.0 mm	0.81	1.12	7.97	16.58	10.17	6.00	0.01	57.34
307109	2.0 mm	0.43	0.41	10.79	16.47	7.37	5.35	0.39	58.79
307113	2.0 mm	0.08	0.53	9.00	14.53	9.31	4.60	-0.01	61.95
307114	2.0 mm	0.16	0.15	7.08	17.75	10.86	5.13	0.11	58.76

[1]CNTRL: nutritionally complete, pelleted herbivorous tortoise diet; 2.0 mm: same tortoise diet with added 2.0 mm length cellulose fiber; 0.2 mm: same tortoise diet with added 0.2 mm length cellulose fiber

[2]S, soluble portion

B. Total Collection Digestibility Data

Table 19. Apparent digestibility (aDig, %) of dry matter (DM), organic matter (OM), neutral detergent fiber (aNDF), and sequential acid detergent fiber (sADF) using total fecal collection of leopard tortoises (*n* = 13) fed one of three nutritionally complete, pelleted herbivorous tortoise diets, exclusively.

		Apparent Digestibility (aDig, %)			
Tortoise	**Diet[1]**	**DM[2]**	**OM[2]**	**aNDF[2]**	**sADF[2]**
307100	CNTRL	89.08	89.83	83.01	83.05
307107	CNTRL	83.94	86.69	78.34	77.54
307110	CNTRL	89.53	90.41	85.22	84.43
307111	CNTRL	87.26	89.76	80.87	80.21
307094	0.2 mm	89.52	90.37	88.59	91.99
307097	0.2 mm	88.65	89.55	86.26	90.56
307098	0.2 mm	86.02	86.88	81.55	86.80
307104	0.2 mm	88.53	89.32	84.48	88.88
307112	0.2 mm	92.97	93.48	90.72	93.16
307101	2.0 mm	92.59	93.11	91.92	93.37
307103	2.0 mm	86.49	87.08	84.52	86.85
307106	2.0 mm	88.65	89.10	85.59	85.82
307114	2.0 mm	87.66	88.19	85.40	87.39

[1]CNTRL: nutritionally complete, pelleted herbivorous tortoise diet; 2.0 mm: same tortoise diet with added 2.0 mm length cellulose fiber; 0.2 mm: same tortoise diet with added 0.2 mm length cellulose fiber

[2]All digestibilities are on a dry matter basis (DMB); DM, dry matter; OM, organic matter; aNDF, α-amylase treated neutral detergent fiber; sADF, sequential acid detergent fiber

C. Acid Insoluble Ash Digestibility Data

Table 20. Apparent digestibility (aDig, %) of dry matter (DM), organic matter (OM), neutral detergent fiber (aNDF), and sequential acid detergent fiber (sADF) using the acid insoluble ash (AIA) marker method of leopard tortoises (*n* = 13) fed one of three nutritionally complete, pelleted herbivorous tortoise diets, exclusively.

		Apparent Digestibility (aDig, %)			
Tortoise	**Diet[1]**	**DM[2]**	**OM[2]**	**aNDF[2]**	**sADF[2]**
307100	CNTRL	88.58	89.37	82.23	82.27
307107	CNTRL	89.58	90.47	84.49	83.91
307110	CNTRL	86.78	87.89	81.34	80.35
307111	CNTRL	90.25	90.86	85.37	84.86
307094	0.2 mm	85.94	87.07	84.69	89.25
307097	0.2 mm	90.42	91.19	88.41	92.03
307098	0.2 mm	90.36	90.95	87.28	90.89
307104	0.2 mm	90.43	91.09	87.04	90.72
307112	0.2 mm	92.82	93.34	90.52	93.01
307101	2.0 mm	90.59	91.25	89.74	91.58
307103	2.0 mm	86.75	87.32	84.81	87.10
307106	2.0 mm	87.27	87.90	83.84	84.10
307114	2.0 mm	88.66	89.15	86.59	92.95

[1]CNTRL: nutritionally complete, pelleted herbivorous tortoise diet; 2.0 mm: same tortoise diet with added 2.0 mm length cellulose fiber; 0.2 mm: same tortoise diet with added 0.2 mm length cellulose fiber

[2]All digestibilities are on a dry matter basis (DMB); DM, dry matter; OM, organic matter; aNDF, α-amylase treated neutral detergent fiber; sADF, sequential acid detergent fiber

D. Fecal Bacterial Phyla Data

Table 21. Fecal bacterial phyla average abundance (%) of leopard tortoises fed one of three pelleted diets, exclusively.

	Average Abundance (%)		
Phyla	**CNTRL[1]**	**2.0 mm[1]**	**0.2 mm[1]**
Bacteroidetes	45.50	42.82	41.16
Firmictues	36.10	38.90	41.10
Spirochaetes	4.94	7.83	4.64
Verrucomicrobia	0.48	1.48	3.44
Lentisphaerae	0.92	3.23	2.08
Tenericutes	1.21	1.00	2.91
Proteobacteria	4.43	2.05	2.23
Elusimicrobia	3.71	0.99	0.40
"Bacteria"	2.10	0.39	0.45

[1]CNTRL: nutritionally complete, pelleted herbivorous tortoise diet (n = 4); 2.0 mm: tortoise diet with added 2.0 mm length cellulose fiber (n = 6); 0.2 mm: tortoise diet with added 0.2 mm length cellulose fiber (n = 6)

E. Fecal Bacterial Genera Data

Table 22. Fecal bacterial genera average abundance (%) of leopard tortoises (*n* = 16) fed one of three pelleted diets, exclusively.

	Average Abundance (%)		
Genera	**CNTRL[1]**	**2.0 mm[1]**	**0.2 mm[1]**
Cytophaga	3.87	16.40	11.91
Bacteroides	9.02	11.48	11.82
Clostridium	16.21	16.64	19.56
Ruminococcus	5.68	7.26	5.19
Treponema	3.95	7.31	4.37
Ruminiclostridium	0.96	3.82	4.59
Anaeroplasma	0.01	0.89	2.71
Solitalea	8.22	5.30	5.99
Akkermansia	0.18	1.33	3.31
Alkaliflexus	11.82	3.90	5.03
Victivallis	0.92	3.23	2.08
Rikenella	2.39	2.44	3.58
Bulleidia	0.22	0.07	1.92
Robinsoniella	0.22	1.52	0.50
Sporomusa	1.41	1.06	0.15
Elusimicrobium	3.71	0.99	0.40
Parabacteroides	4.82	1.29	0.76
Eubacterium	0.07	1.25	1.03
Oscillospira	0.55	1.43	0.82
Anaerostipes	0.21	0.30	0.92
Syntrophomonas	0.99	0.63	0.12
Nitrosovibrio	2.37	0.98	1.00
Haloplasma	2.10	0.39	0.45
Dehalobacterium	0.83	0.55	0.20
Lachnoclostridium	0.45	0.87	1.08
Lachnospira	1.37	0.75	0.67
Barnesiella	0.00	0.40	0.77
Petrimonas	0.00	0.51	0.20
Turicibacter	0.00	0.26	0.40
Alistipes	2.13	0.34	0.61
Candidatus armantifilum	1.19	0.30	0.14
Planctomyces	0.00	0.37	.0.41
Synergistes	0.00	0.20	0.43
Haloplasma	2.10	0.39	0.45
Arcobacter	1.30	0.02	0.09
Acholeplasma	1.19	0.10	0.20
Hespellia	0.62	0.13	0.11
Acetovibrio	0.66	0.23	0.14
Bacillus	0.60	0.15	0.14
Leptospira	0.52	0.21	0.07

[1]CNTRL: nutritionally complete, pelleted herbivorous tortoise diet (*n* = 4); 2.0 mm: tortoise diet with added 2.0 mm length cellulose fiber (*n* = 6); 0.2 mm: tortoise diet with added 0.2 mm length cellulose fiber (*n* = 6)