FEDERAL RURAL UNIVERSITY OF PERNAMBUCO
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STABLE ISOTOPES IN THE RUMINANT DIETARY IDENTIFICATION

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RECIFE-PE

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Thesis presented to the graduate program in animal science of Federal Rural University of Pernambuco, required to obtain, partially, the Master degree of sciences.

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SUMMARY

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Utilization of carbon stable isotopes in the ruminant dietary identification

Introduction

Animal production is based on nutrition. Pastures are the major components in many livestock production systems, and in most cases, they are solely composed by grass. As an alternative to reduce the costs, mitigate the impacts of inorganics fertilizers, and improve forage quality, the combination of grass and legume in the pasture, has been used around the world (Muir et al., 2011; Lüscher et al., 2014). In addition, legumes can, also, improve the digestibility of grasses, when consumed in legume-grass mixtures (Muir et al., 2014).

The forage consumption is very important to animal production. There are several methods to determine the forage intake, and many of them are based on total fecal output and forage digestibility (Minson, 1990; Burns et al., 1994). The forage digestibility is also an important factor to estimate the intake, thus the digestibility assessment has to be accurate, which is not always an easy task in grazing trials.

When grazing occurs in grass-legume mixed pastures, digestibility determination is more challenging because of animal selection. Different proportions of grass and legume affect directly the digestibility. Stable isotopes have been used as a tool, for decades, to find out the digestibility of mixed diets (Jones et al., 1979; Martinez del Rio et al., 2009; Wolf et al., 2009). The carbon stable isotope ratio (^{13}C:^{12}C) can be used to identify plants that have different photosynthetic pathway, such as C_3, C_4, and CAM, and can be used to evaluate their presence in the diet, by analyses of the feces (Martinez del Rio et al., 2009).

Legumes and tropical grasses have different $\delta^{13}C$. The grass has a $\delta^{13}C$ value range of -9‰ to -17‰, and the legume from -20‰ to -32‰ (Ballentine et al., 1998). These values can be used to identify the proportions of legume and grass in the feces. Therefore, it is possible to estimate the proportion of C_3 and C_4 species consumed by livestock grazing grass-legume

pastures. Fecal $\delta^{13}C$ values are often more depleted than the dietary values because of discrimination against the heavy isotope. The use of feces and diet to find the proportions of legume and grass need to be validated because it is species specific.

The objective of this research is to validate the use of the carbon stable isotope ratio ($\delta^{13}C$) to determine the proportions of C_3 and C_4 species in the diet based on fecal $\delta^{13}C$ determination, evaluating the prediction models using indigestible neutral detergent fiber (iNDF) procedure and untreated samples.

Literature Review

1. Grassland

Grassland is the land and the vegetation growing on it devoted to the production of introduced or indigenous forages, including grasses, legumes and other forbs, and at times other woody species (Allen et al., 2011). Grasslands are one of the largest terrestrial agroecosystems. They cover approximately 40.5 percent of the terrestrial area on Earth, except the poles, equating to 52.5 million km^2. Grasslands provide ecosystem services (ES) such as provision of forage for domestic livestock, carbon (C) sequestration, water catchment and filtration, habitat for wildlife, nutrient cycling, and many others (Dubeux et al., 2014). Provisional ES have a direct economic impact, benefiting many millions of farmers to produce meat, milk, wool and other animal commodities (White et al., 2000). Forage obtained from grasslands provides feed and nutrients to animals at lower cost than concentrate feeds, though forages can vary in nutritive value due to differences in species, environment, and timing of harvest (Givens et al., 2000). In many cases, it is more economically feasible to let animals graze rather than to provide supplementation, since profits may be reduced by providing supplements (Redmon & Hendrickson, 2007).

Grazing can potentially be a sustainable activity that does not burden the environment. Specific subsystems such as sowed pasture and natural pasture need special attention to avoid losses in economic and environmental contributions due to negative management (Rótolo et al., 2007). Intensification of the grazing system is generally linked to nutrient inputs via commercial fertilizers and supplement feed to animals. Excess of nutrients deposited in intensive grazing systems by fertilizer and animal wastes can cause environmental disturbance. Areas where animals congregate such as feedlots and dairy farms are even more problematic

regarding nutrient losses to the environment (Vendramini et al., 2007). In conventional grass-only pastures, the grass production depends on the use of synthetic nitrogen (N) fertilizer, in order to provide a high-quality forage, but this supply has a cost, that influences cattle production. Incorporating legumes into grass pastures has potential to supply N, reducing production costs (Butler et al., 2012). Some economic and environment factors affect pasture management. Rational use of grasslands and grass-legume mixtures are an important option to improve management (Muir et al., 2011).

2. Mixed grass-legume pastures

2.1 Legume pasture

Improvement of soil N and the potential increase on sward production with reduced N fertilizer application are advantages of using legumes in grazing systems, considering that legumes are recognized as a source of biologically fixed N (Ledgard & Steele, 1992). These facts are essential to understand the role of legumes in the production system and their contribution to ruminant livestock production. Due to their nutritive value and ability to fix atmospheric N_2 via association with soil microorganisms, legumes have been used in many grassland systems around the world (Rochon et al., 2004). Ledgard (2001) reported that many studies indicate inputs of 200 to 400 kg N ha^{-1} yr^{-1} by forage legumes to the soil-plant-animal. Field studies, however, demonstrate limitations that reduce this potential fixation to 20 to 200 kg N ha^{-1} yr^{-1}. Rotz et al. (2005) obtained mean annual N_2-fixation rates of white clover ranging from 0 to 166 kg N ha^{-1}. Cadisch et al. (1994) considered sufficient to sustain the productivity of the mixed pasture system 60 to 117 kg N ha^{-1} $year^{-1}$, and reported that the inclusion of persistent legumes can improve the sustainability of pasture production.

Industrial fertilizers contribute indirectly to enhance soil C via increase in primary productivity; however, during the manufacturing process of fertilizers, there is a release of CO_2

to atmosphere (Schlesinger, 2009). Pastures with 25% of nodulating legumes in their botanical composition fixing 60 kg N ha^{-1} $year^{-1}$ were equivalent to pastures receiving 100 kg N ha^{-1} $year^{-1}$ of inorganic fertilization (Lira et al. 2006). Mixed grass-legume systems, can contribute with 10-75 kg N ha^{-1} $year^{-1}$ to grass, via legume (Nyfeler et al., 2011). The establishment of N-fixing legumes provides benefits, such as improvement of soil organic matter (Deyn et al., 2011). Therefore, including forage legumes and reducing the use of industrial N fertilizer will mitigate the carbon footprint of livestock production systems, contributing to reduce the greenhouse effect.

Legumes contribute to enhance soil fertility and increase pasture production. Furthermore, they can also be an excellent source of N for animal nutrition, depending on their nutritive value. Sleugh et al. (2000) evaluated seasonal yield distribution and forage quality of grass-legume mixtures. They observed that grass-legume mixtures can improve the nutritive value and seasonal distribution of forage yield, reducing the need for livestock supplementation. Cantarutti et al. (2002) evaluated the effect of a legume (*Desmodium ovalifolium*) in swards of *Brachiaria humidicola.* The presence of legume significantly increased herbage N concentration and N recycled via litter deposition, ranging from 42 to 155 kg N ha^{-1} $year^{-1}$ for 4 and 2 head ha^{-1}, respectively. Muir et al. (2011) performed a meta-analysis and observed that, for warm-season grass, minimum crude protein (CP) concentration was 78 ± 9 g kg^{-1} and for legume was 151 ± 9 g kg^{-1}. They concluded that the most important nutritional contribution from legumes to grass-legume mixture is to provide CP for ruminants. In grass-legume mixtures, grazing animals can build their diet selecting different forage species and varying nutrient concentration, reaching their nutritional requirement (Gregorini et al., 2015).

3. Intake

3.1 Forage intake

Feed intake is essential for animal nutrition, in order to ingest the necessary nutrients for maintenance and performance (Van Soest, 1994). Intake is defined by Mertens (1994) as the absolute amount of dry matter (DM) ingested per unit of time. Intake is often measured over a period of 5 to 10 days, and it is expressed as daily quantity per unit of body weight (BW). Intake and digestibility are considered the major components determining ruminant production (Mertens, 2007).

Factors such as animal, forage, and environmental conditions can control forage intake. Under grazing, however, some factors are unique, such as preference, bite size, water content of forage, herbage mass, herbage accumulation, herbage allowance, and sward heterogeneity (Minson, 1990; Minson and Wilson, 1994). Grazing factors can influence the intake measurement, due to the possibility of animals to select parts of the plant or plant species. Selection indicate preferential consumption of a feed subcomponent, such as parts of plant and plants within a specific physiological state (Mertens, 1994). Through selective grazing, animals are free to search for feed and select the diet, increasing its nutritional diet. In order to achieve this goal, they develop their own feeding strategy (Baumont et al., 2000).

The measurement of DM intake (DMI) for grazing animals can be laborious and time consuming. Handling the animal intake and the fecal output collection are more difficult to accomplish in pastures than housed animals. As a result, these measurements, under grazing conditions, are hard to obtain with accuracy (Van Soest, 1994; Gregorini et al., 2015). In mixed pastures and rangelands, the problem might become worst because of the heterogeneity of the vegetation (Oltjen et al., 2015). Estimating diet selection is not a simple task in complex grazing situation, such as rangelands, with several forage species (Baumont et al., 2000).

3.2 Methods to estimate forage intake

3.2.1 Direct methods

Minson (1990) described methods for intake determination, such as short-term change in live weight, cutting method, grazing behavior, and fecal techniques. These techniques are classified as direct and indirect measurements. Short-term change in live weight, or difference in animal mass is a direct method, that has been often used to determine the intake of forage in grazing animals. This method is based on the difference in weight of animals before and after grazing, considering losses in weight by feces and urine (Minson, 1990; Burns et al., 1994).

Difference in herbage mass and pre/post grazing is not used frequently to estimate forage intake (Burns et al., 1994). Herbage disappearance was defined by Macoon et al. (2003) as a method to predict forage DMI by calculation of difference between pre-grazing and post-grazing herbage mass. Animal daily intake is represented as the relationship between herbage mass disappearance after grazing, the number of animals, and grazing period (Burns et al., 1994). Macoon et al. (2003) concluded that the herbage disappearance method was suitable for their study.

Satiation and motivation to eat are key aspects in grazing behavior (Baumont et al., 2000; Forbes & Gregorini, 2015). The method is based on grazing period, bite mass, and bite rate; it is considered suitable to estimate short-term (i.e. 15 to 30 min) forage intake (Minson, 1990). Estimate of accurate bite size (i.e. DMI per bite) is considered the limiting factor for this method (Burns et al., 1994). Another flaw of this method is to base long-term measures (forage intake) on short-term measures (bite rate and bite size). Errors might occur when scaling up from short-term to long-term. Laca et al. (2000) calculated the bite mass (BM, g $bite^{-1}$) using forage DM, fresh weight of pre- and post-grazing, weight of losses (evapotranspiration) of forage used, and the number of bites taken. The authors also evaluated the sounds (videotape) provided by grazing animals. They concluded that analyses using

grazing sounds can solve some problems related with measurements of grazing intake. Forbes and Gregorini (2015) presented feed intake (g day^{-1}) as a function of meal frequency (meals day^{-1}) and meal size (g $meal^{-1}$).

3.2.2 Indirect methods

A variety of indirect and complex techniques has been evolved, due to difficulty of measuring forage DMI in grazing animals (Burns et al., 1994; Van Soest, 1994). In past decades, the technique to estimate the forage intake of grazing animals by determining total fecal output was described. The method is simple, using bags to collect feces and the apparent digestibility of the forage, obtained by animals in feedlot (Minson, 1990). In order to calculate the DMI of grazing animals, it is necessary to know the fecal output (FO) and digestibility. The equation to estimate DMI was described by Minson (1990) and Carvalho et al. (2007) as:

$$\text{Intake (g DM/day)} = \text{Feces output}/(1\text{-DM forage digestibility})$$

3.2.3 Fecal output estimate

There are two ways to obtain the fecal output, direct and indirect. The direct way involves total fecal collection using collection bags placed on animals. Indirect estimate using markers is less invasive. Burns et al. (1994) indicated that total fecal output can be estimated by the ratio between the quantity of a marker dosed to an animal and its concentration in the feces.

Markers are classified as internal or external, due to the difference of nature of the compound and application, because, the external marker needs to be ingested by the animal, which is an invasive method. External markers are indigestible components added in the diet

to be retrieved from the feces, or dosed in the animal. This method is indicated for animals under grazing conditions. Examples of external markers used in studies include, *n*-alkane, chromium oxide, polyethylene glycol (PEG), and LIPE® (Carvalho et al., 2007; Azevedo et al., 2014; Benvenutti et al., 2014). Pulse-dose marker is a method that permit the inert marker application just once, with frequent collections thereafter (Burns et al., 1994; Macoon et al. 2003).

Chromium-mordant fiber is another approach to use chromium as a marker. Hand-plucked forage samples are necessary to simulate the grazing and represent the forage used as source of fiber to bind with the chromium (Macoon et al., 2003). The *n*-alkanes are used as an internal (plant) and external (dosed) marker to provide an estimate of diet composition, forage intake, and fecal production (Dove and Mayes, 2005). The *n*-alkane marker was used by Azevedo et al. (2014), and reported the importance of a rigorous methodology to collect samples, especially from pasture, in order to obtain a representative plant portion, as well as the fecal estimative. The daily-dose marker is a method in which markers are administered twice per day; it is considered a laborious technique, and can cause stress on animals (Burns et al., 1994). Daily doses are applied according to the animal weight, approximately 0.5 to 1 g for sheep and 5 to 10 g for cattle (Carvalho et al., 2007). Feces collection to determine marker concentration can be conducted directly from the animal, by rectum grab, during weighing, milking, or any other routine procedure with the animals. Another approach to collect the feces is by observing animals in the pasture and collecting fecal samples right after defecation (Macoon et al., 2003).

Internal markers are compounds found in the feed that are indigestible. Fecal protein, indigestible dry matter (iDM), indigestible neutral detergent fiber (iNDF), and indigestible acid detergent fiber (iADF) are examples of internal markers that have been used in many studies

(Azevedo et al., 2014; Casali et al., 2008). The advantage of the use of internal markers is the reduction of animal stress, because it is not necessary the administration of marker and have been used in feedlot trials.

3.2.4 Forage digestibility

Forage digestibility affects fecal output and it is necessary to estimate forage intake. Accurate measurement of digestibility is crucial to predict DMI (Barnes et al., 1994), however, representative samples from grazed pastures are difficult to obtain. Cattle prefer plant portions and plant species in a unique way. Collecting representative diet samples in mixed pastures might become even more problematic, because of cattle preference. Regardless of the forage species or the region (tropical or temperate), the digestibility of the forage in the diet selected by grazing animal usually is 60% greater than the digestibility of the total herbage mass. Thus, error from forage digestibility estimate as a result of inaccurate sampling affect more the forage intake estimate than the error from the fecal output determination (Carvalho et al., 2007). Dove (1996) also reported problems related to herbage intake estimation due to potential errors from in vitro digestibility results.

Macoon et al. (2003) determined forage digestibility on composite hand-plucked samples collected from 15 to 20 random sites within each grazed pasture. In order to reduce the forage sampling error, forage samples should be hand-plucked or collected using esophageal fistula (EF) (Carvalho et al., 2007). The ingesta, however, represents the botanical composition of the diet consumed during a short grazing period, but the analysis based on fecal samples allows to assess diet botanical composition over days (Dove, 1996).

Bennett et al. (1999) used the micro histological technique (MH) by cannulated animals and stable carbon isotope ratio (SCIR) to determine botanical composition on the diet consumed by animals grazing legume and grass. They concluded that both methods could define the botanical composition. In mixed pastures, the botanical composition by hand-

plucked samples can be very different from the diet selected by grazing animals, because the animal consumes specific proportions, not represented by hand-plucked samples. Therefore, the use of SCIR have been recommended, once that the proportion of the plant selected by animal is presented in the feces. Dove (1996) reported that SCIR have proved useful to identify plant photosynthetic pathway. Thus, in pasture with a mixture of C_3 and C_4 plants, in which it is difficult to estimate the animal selectivity, SCIR can be a tool to estimate the botanical composition of the animal diet, using fecal samples.

4. Stable Isotopes

4.1 Stable Isotope Definition

As a reference to the periodic table, the word "isotope" comes from Greek and means that one isotope of an element occupies the same (*iso*) place (*topos*) in the periodic table (Dawson and Brooks, 2001; Fry, 2006). Thus, isotopes are defined as the same element that differ in number of neutrons in the nucleus; they can be stable and radioactive (Fry, 2006; Sulzman, 2007).

The ^{13}C isotope, for example, has 6 protons and 7 neutrons, while the ^{12}C has 6 protons and 6 neutrons, and both carbon isotopes are used for the same purpose and have the same functions. Therefore, the atomic weight is different, due to the extra neutron. This extra neutron makes the nucleus heavier, but does not affect most of its chemistry (Fry, 2006). The heavier molecules or ions have a stronger bond, so more energy is necessary to break it, and they react slower than the lightest ones (Freitas et al, 2010).

4. 2. Isotope Notation

The isotope ratios have been expressed by delta (δ) notation, which is the difference, relative to internationally accepted standards (Fry, 2006). Because of the high price, one or more internal working standards are used in the laboratories, which are compared against the

international standard (Ehleringer and Rundel, 1989; Sulzman 2007). The standard for hydrogen and oxygen is Standard Mean Ocean Water (SMOW), for carbon, it is a fossil, the PeeDee Belemnite (PDB), for nitrogen it is air (AIR) and for sulfur it is Canyon Diablo meteorite (CD) (Ehleringer and Rundel, 1989; Hayes, 2004; Fry, 2006).

The δ values are commonly expressed in per mil (‰) (Tcherkez et al., 2011), which is not a unit, but actually a deviation from the ratio of heavy to light isotopes in the sample by the same ratio from the standard, considered to have δ value equal to 0. The symbol ‰ (permil, from the Latin *per mille* by analogy with *per centum,* percent, 10^{-3}*)* is used to simplify, and it implies the factor of 1000, which is equivalent to express either δ -25‰ or δ -0.025 (Farquhar et al., 1989; Dawson and Brooks, 2001; Hayes, 2004). The δ calculation is summarized in the following equation:

$$\delta^{M}E = \left[\left({R_{Sample}}/{R_{Standard}}\right) - 1\right] * 1000$$

Where E denotes the element, M is the mass of the heavy isotope, R is the ratio of the heavy to light isotope. Thus, the ratio of ^{13}C:^{12}C is expressed as $\delta^{13}C$ and ^{15}N:^{14}N becomes $\delta^{15}N$ (Ehleringer & Rundel, 1989; Dawson & Brooks, 2001; Crawford et al., 2008). The final δ value is expressed as the amount of the rarest to commonest isotope in the sample, the higher δ values indicate greater proportion of the least common isotope (Dawson et al., 2002).

The natural abundance is the range of δ between -100 and +50‰ for natural samples. Samples with greater proportion of the least common isotope in relation to the standard, are commonly referred to as being ‘enriched’, and samples with proportionally less are referred to as ‘depleted’ (Dawson et al., 2002; Fry, 2006; Crawford, 2008).

278 *4. 3. Isotope measurement*

279 Isotope abundance in any sample, enriched or not, is measured, with precision, using a
280 isotope ratio mass spectrometer (Dawson et al., 2002), after chemical derivatization (Peterson
281 and Fry, 1987; Fernandez et al., 1996). The mass spectrometer was invented by J.J. Thompson
282 in 1910 (Sulzman, 2007), and it is an instrument which generates ions from either inorganic or
283 organic compounds, and separate these ions by their mass-to-charge ratio (*m/z*), detecting them
284 qualitatively and quantitatively by their respective *m/z* and abundance (Gross, 2004). Most
285 mass spectrometers can measure only low molecular weight compounds, usually with molecule
286 mass less than 64 (Ehleringer and Rundel 1989). Current-generation isotope ratio mass
287 spectrometers (IRMS) have three or more Faraday cups, positioned to capture specific masses
288 (e.g., 44, 45, 46) simultaneously (Sulzman, 2007).

289 The IRMS is required for accurate detection of small differences and gaseous samples
290 required for the isotopic determinations (Peterson and Fry, 1987; Dawson and Brooks, 2001).
291 The objective of this analysis is to convert a sample quantitatively to a suitable purified gas
292 (typically CO_2, N_2, or H_2) that the IRMS can analyze. These samples are usually organic, which
293 must be initially dried and ground to a fine powder (Michener and Lajtha, 2007), and then
294 combusted until it emerges as a simple gas (Fry, 2006). The samples are introduced into the
295 IRMS via inlet system as a gas, under ambient conditions, where would naturally move into
296 the IRMS by molecular flow and if they were of different masses, fractionation would occur
297 (Dawson & Brooks, 2001). The difference in the signal between the sample and the standard
298 gases is used to calculate the isotope ratio for the sample (Ehleringer and Rundel, 1989).

299 The IRMS consists of an inlet system, an ion source, an analyzer for ion separation,
300 and a detector for ion registration (Brand, 2004). There are two types of IRMS, the dual-inlet
301 (DI-IRMS) which has a higher precision and the continuous flow (CF-IRMS). In the

continuous flow, it is possible to introduce multiple component samples (atmospheric air, soil, leaves), and obtain isotopic composition for individual elements or compounds within the mixture; all differences between them are in the inlet component (Sulzman, 2007). The DI-IRMS requires a skilled operator, takes more time to run one sample than CF-IRMS, and are more expensive (Dawson and Brooks, 2001). Most ecologists currently use dual CN isotope measurement made with elemental analyzers coupled to mass spectrometers (Fry, 2006).

4. 4. Isotope samples

Many materials can be used, such as wool, blood (Kristensen et al., 2011; Martins et al., 2012; Norman et al., 2009), food (Deniro and Epstein, 1978; Hwang et al., 2007; Martins et al., 2012; Norman et al., 2009), milk (Braun et al., 2013), soil (Hall and Penner, 2013), plant material (Ballentine et al., 1998; Fernandez et al., 2003; Gessler et al., 2008), minerals (Michener and Lajtha, 2007), seawater (Peterson et al.,1985; Fry, 1991), tooth, bone collagen (Sponheimer et al.,2003), and feces (Jones et al., 1979; Botha and Stock, 2005; Hwang et al., 2007; Norma et al.,2009; Kristensen et al., 2011; Martins et al.,2012). Many elements have two or more naturally occurring stable isotopes (Crawford et al., 2008) and are used in isotope analysis, such as carbon (C), nitrogen (N), sulfur (S), hydrogen (H) and oxygen (O), where CNS elements are more related to organic matter cycling and HO elements are more related to the hydrological cycle (Fry, 2006). The use of C, N, O, and H to study physiological processes has increased exponentially in the last thirty years (Marshall et al., 2007).

4.5. Isotope technique and field analysis

Stable isotope chemistry was once the domain of the earth sciences, but the use was largely inaccessible to biologists, due to difficulty with the technique. The situations has changed in the past two decades with the improvement of the tool (Martínez del rio et al., 2009). Stable isotopes may serve as potentially useful markers of ecosystem process studies

(Ehleringer and Rundel, 1989; Brazier, 1997). They might also be useful in studies of animal ecology (Gannes et al., 1997) and geochemistry cycles (Freitas et al., 2010).

Access to isotope ratio mass spectrometers has increased and the costs for sample analysis have decreased in recent years. As a result, researchers of different fields have increasingly added stable isotope analysis as additional tool in their investigation (Michener and Lajtha, 2007). Stable isotope analyses have an important contribution to animal ecology and food chains. The foods that animals eat often shows specific isotopic composition (Gannes et al., 1997). Carbon isotopes can be used to back calculate the diet of small ruminants (Norman et al., 2009), cattle (Jones et al., 1979), and wild herbivores (Botha and Stock, 2005). Likewise, ^{15}N in feces can be useful to reconstruct animal diet (Hwang et al., 2007) and the sulfur isotope ^{34}S as an additional tool for salt marshes and estuaries studies (Peterson et al., 1985). Stable isotopes have been also used to identify sources of pollutants, heterotrophic nitrification, and to estimate rates (e.g. soil C turnover). They can also be used to evaluate models derived from other techniques, to corroborate, reject, or restrict results from other analyses (Sulzman, 2007).

5. Carbon Stable Isotopes

The carbon cycle is defined by exchanges of CO_2 between atmosphere and terrestrial ecosystems (Fry, 2006). In the nature, there are two stable isotopes of carbon, the ^{12}C and ^{13}C; the ^{12}C corresponds to approximately 99% of the total C, while the ^{13}C about 1% (Ludlow et al., 1976; O'Leary et al. 1988, Farquhar et al., 1989).

The $\delta^{13}C$ of atmospheric CO_2 is decreasing due to inputs of depleted CO_2 from fossil fuel burning and decomposition (Fry, 2006). In the absence of industrial activity, the $\delta\ ^{13}C$ value of atmospheric CO_2 is -8‰ (O'Leary et al.1988). Plants contain less ^{13}C than the atmospheric CO_2 on which they depend for photosynthesis; therefore, plants are "depleted" of ^{13}C relative to the atmosphere. This occurs because enzymatic and physical processes

discriminate against ^{13}C in favor of ^{12}C (Marshall et al., 2007; Farquhar et al., 1989). Different discrimination occurs between plant physiological groups. This information can be used to specify plants that use different photosynthetic pathways (O'Leary et al., 1988). Stable carbon isotopes (^{12}C and ^{13}C) at natural abundance is a tool to assess physiological, ecological, and biogeochemical processes related to ecosystems (Tcherkez et al. 2011).

5. 1. Stable Isotope Carbon Natural Abundance

The C_3 plants, that photosynthesize exclusively via the Calvin photosynthetic cycle, and C_4 plants that have C_4 carbon cycle are different in leaf anatomy (Taiz and Zeiger, 2002) and have different photosynthetic pathways. Therefore, ^{13}C discrimination varies between these two physiological groups (Marshall et al., 2007). Soil organic matter is depleted in ^{13}C compared to atmospheric CO_2 and the standard. Ratios are reported in the differential notation relative to the PDB standard (Focken, 2004), that is a Cretaceous belemnite, *Belemnitella americana*, from the Pee Dee Formation of South Carolina (Craig, 1957; Lerman,1975; Hayes, 2004). Because the PDB is no longer available, a new reference standard, Vienna-PDB (VPDB) has been defined (Sulzman, 2007), and recent reports often present values of δ_{VPDB} equal to values of δ_{PDB} (Hayes, 2004).

In the literature, it is possible to find a variety of $\delta^{13}C$ signatures. O'Leary et al. (1988) mentioned the $\delta^{13}C$ value of C_3 near -28‰ and C_4 approximately -14‰, while Fernandez et al. (2003) reported the value of C_3 ranging from -26‰ to -29‰ and the C_4 plants commonly ranging from -12‰ to -14‰. Ballentine et al. (1998) observed a variety of C_3 plants with $\delta^{13}C$ value ranging from -20‰ to -32‰, and the C_4 plants from -9‰ to -17‰. Thus, the ^{13}C composition in photosynthetic products vary between plants species, plant developmental stages, and environmental conditions (Ghashghaie and Tcherkez, 2013).

374 *5. 2. C_3 and C_4 plants $^{12}C/^{13}C$ discrimination*

375 The atmospheric CO_2 is transported through the boundary layer and the stomata into
376 the internal gas space to dissolve in the cell, and then diffuses to the chloroplast, where the
377 carboxylation occurs (O'Leary et al., 1988). The atmospheric CO_2 transported is composed of
378 ^{12}C and ^{13}C. The intra cell diffusion has an apparent fractionation ($\Delta\delta$) of about 4.4‰ due to
379 the slower motion of the heavier ^{13}C (Marshall et al., 2007). The atmospheric CO_2
380 transportation process is reversible; however, the carboxylation step is irreversible and after
381 this event the isotope fractionation does not change (O'Leary et al.,1988). If diffusion
382 exclusively limits photosynthesis and the stomatal resistance is high, the fractionation would
383 reflect only the diffusive processes, where the $\Delta\delta$ is about 4‰. Whether the diffusion has no
384 limitation, the stomatal diffusion is quick, the fractionation would be equivalent to the
385 enzymatic step, then the $\Delta\delta$ is about 29‰ (Farquhar et al., 1982; O'Leary et al., 1988; Marshall
386 et al., 2007).

387 The CO_2 diffusion in air and aqueous solution have a small fractionation, $\Delta\delta$ 4.4 and
388 0.7‰ and the enzyme ribulose bisphosphate carboxylase/oxygenase (rubisco) discriminates
389 against the ^{13}C and with a fractionation of 29‰ (O'Leary et al.,1988; Marshall et al., 2007). In
390 C_3 plants, CO_2 uptake is more limited by the rate of rubisco than by diffusion (O'Leary et al.,
391 1988). The average total organic matter is depleted in ^{13}C by nearly 20‰ compared with
392 atmospheric CO_2 (Tcherkez et al., 2011). Thus during photosynthesis, a fractionation of 20‰
393 occurs between the source atmospheric CO_2 at -8‰ and the -28‰ plant sugar product, that is
394 formed from atmospheric CO_2 (Fry, 2006).

395 For C_4 plants, the $\Delta\delta$ is about 4‰ due to the involvement of the CO_2-concentrating
396 mechanism involving the phosphoenolpyruvate carboxylase (PEPc) (Ghashghaie & Tcherkez,
397 2013). The CO_2 diffuses through stomata to the mesophyll cells, where it dissolves and is

converted to bicarbonate (HCO_3^-), which is in equilibrium with CO_2 in ^{13}C concentrations (O'Leary, 1988; Farquhar et al., 1989). Thus, the isotopic fractionation in C4 is the result from the fixation of ^{13}C enriched HCO_3^- by PEPc. Therefore, the products from the Calvin cycle simply reflect the net effect of CO_2 fixation by the PEPc and are about 4‰ depleted compared with atmospheric CO_2 (Tcherkez et al., 2011), thereby the predicted $\delta\ ^{13}C$ value is -12 ‰. The steps that are significant for isotope fractionation are stomatal diffusion and PEPc, where the diffusion is the first limiting in C_4 plants (O'Leary,1988).

6. Carbon stable isotopes and the reconstruction of animal diets

6. 1. Carbon analysis technique for animal diet

The carbon stable isotope method is based on fractionation of ^{13}C by plants in the photosynthesis pathway (Botha and Stock, 2005), and has been used to provide a quantitative description of the diet where different sources can be analyzed from a single sampling (Crawford et al., 2008). Diet isotopic composition can be similar as that of animal tissues (Gannes et al., 1997). Materials such as hair, blood, and feces are suitable, due to non-destructive collection (Hwang et al., 2007), and provide dietary information with different temporal scale. Fecal samples from animals that are fed with different proportions of C_3 and C_4 plants reflect short-term dietary changes and the collection is easier than plasma samples (Norman et al., 2009), allowing to assess diet selectivity over short-time scales (Botha and Stock, 2005).

Fecal $\delta^{13}C$ changes within few days of consumption, whereas the changes in hair samples $\delta^{13}C$ provides longer-term assessment (Sponheimer et al., 2003a; Martins et al., 2012). Therefore, this technique has been used to study diet selection by animals. Analysis of feces can be an irreplaceable tool for field ecologists to study diet variations (Codron & Codron,

2009). The use of wool and feces constitutes an easy and non-invasive approach to examine wild herbivore diet in protected areas (Kristensen et al., 2011).

6. 2 Back calculate the animal diet

The SCIR is useful to estimate the proportion of dietary sources on mixed diets (Ludlow et al., 1976). Thus, the proportion of legume and grass ingested in the feed or pasture can be calculated via $\delta^{13}C$ of feces, using the $\delta^{13}C$ of C_3 plants (legume) and C_4 plants (grass) (Jones et al., 1979). DeNiro and Epstein (1978) reported that it is possible to perform dietary analysis based on $\delta^{13}C$ value. Bennett et al. (1999) estimated the diet botanical composition in cattle grazing mixed pasture of C_4 grass and C_3 legume, analyzing the $\delta^{13}C$ of feces. Sponheimer et al. (2003) corroborated the idea by stating that the stable carbon isotope technique is applicable and efficient to quantify relative proportions of graze and browse in an animal diet. Samples from bovine muscle are efficient to distinguish beef origin, from pasture, concentrate, or different proportions of dietary components (Osorio et al., 2011).

In summary, the technique is frequently used to back-calculate proportion of feed sources in the diet (Focken, 2004; Hwang et al., 2007). Using fecal and dietary samples, Jones et al. (1979) developed a technique to estimate the proportion of C_3 and C_4 selected by grazing animals using $\delta^{13}C$. Norman et al. (2009) used equations to estimate the proportion of forage types on intake by sheep, considering the fractionation between diet/tissue, and the organic matter digestibility and indigestibility to improve the accuracy of the prediction. Macedo et al. (2010) predicted the proportion of *Desmodium ovalifolium* (legume) mixed with *Brachiaria dictyoneura* (grass) in the diet for confined cattle, compared with known intake proportions. Martins et al. (2012) evaluated the carbon turnover for sheep and found values that indicated the type of diet fed, based on C_3 and C_4 plants.

Likewise, Focken (2004) back-calculated the proportion of mixed diet of fish (controlled condition) via linear interpolation between the fish on the two sources used, compared to the condition in which it was applied individually. Bruckental et al. (1985) determined the digestibility of hay (C_3) and grain (C_4), individually, used to feed rams in different proportions of the diet, using fecal $\delta^{13}C$ value.

6. 3. Difference between dietary and animal tissue $\delta^{13}C$

The isotopic reconstruction of the diet from $\delta^{13}C$ value from animal tissue or feces is based on the hypothesis that there is a known constant relationship between the $\delta^{13}C$ of the forage and the tissue or feces (Wittmer et al., 2010). Norman et al. (2009) found a positive relationship between $\delta^{13}C$ values of samples, such as feces, plasma, rumen solids, rumen liquor, urine and wool and the $\delta^{13}C$ of the diet. However, the animal isotopic signal incorporation rate from the diet differs among organisms and tissue, individually (Martínez Del Rio et al., 2009; Wolf et al., 2009). Different digestibility or fractionation during assimilation and metabolic processes can change the stable isotopic relationship between diet and animal (tissue) (Gannes et al. 1997; McCutchan Jr et al. 2003, and DeNiro and Epstein 1978). This relationship depends on both the type of tissue and the nature of the diet. The accuracy of this relation is limited by the seasonal variation of $\delta^{13}C$ of the diet, and the random intake of plants in the field (DeNiro and Epstein 1978).

6. 3. 1. Diet-animal sample discrimination

The isotopic difference between tissue and diet is known as tissue-diet discrimination, and is presented as Δ ($\Delta = \delta_{tissue} - \delta_{diet}$) (Wolf et al., 2009). Some authors present the calculations, models or expression based on tissue, but this can be also applied to feces (Jones et al., 1976; Botha and Stock, 2005; Hwang et al., 2007; Norma et al., 2009; Kristensen et al., 2011; Martins et al., 2012), ruminal fluid (Norman et al., 2009), and breath (CO_2) (Ayliffe et

al., 2004; Passey et al., 2005). The $\delta^{13}C$ information is related to the type of animal sample or product analyzed; for instance, feces or samples from gut represent the diet information in a short-time scale, while animal tissue represents the long-term scale (Tieszen et al., 1983).

The relationship determined by DeNiro and Epstein (1978) between the $\delta^{13}C$ of the animal and the diet ingested, was about 1‰ compared with the diet, where the $\delta^{13}C$ value obtained from the whole animal was considered enriched. Wittmer et al. (2010) assessed the relationship among forage, feces, and wool of sheep under grazing. They found that the $\delta^{13}C$ of feces and diet was 0.6‰, feces and wool -4.3‰, as well as wool and diet -3.9‰. In feces, Jones et al. (1976) obtained -0.4 (grass) and -2.0 ‰ (legume) for $\delta^{13}C$ between herbivore diets and fecal samples. Sponheimer et al. (2003) also examined the $\delta^{13}C$ of diet-feces for herbivores fed with alfalfa and bermudagrass (*Cynodon dactylon*) and observed the mean $\delta^{13}C$ of -0.8‰ for both diets, where the alfalfa (-0.6‰) $\delta^{13}C$ was less depleted than bermudagrass (-1.0‰). The $\delta^{13}C_{\text{diet-feces}}$ found by Norman et al. (2009) was -0.94‰ for sheep fed on plants with C_3 and C_4 photosynthetic pathways.

Some authors have assumed this discrimination as constant to estimate the proportion of different sources in the diet (e.g., Sponheimer et al., 2003; Codron et al., 2007, 2011; Norman et al, 2009). The diet–tissue discrimination factors can vary. Thus, using fixed discrimination factors, and (or) discrimination factors that are not diet-dependent, obtained from literature, might result in error in the determination of mixed diet composition (Caut et al., 2008).

6. 3. 2. Fecal endogenous contamination

The variation between $\delta^{13}C$ value of feces and diet might also be explained by endogenous contamination, as tissue or fluid from the gut is expelled and results in an over-estimation of the quantity of a source in the diet, due to the fact that the $\delta^{13}C$ is different (more depleted) from the $\delta^{13}C$ value ingested (Jones et al., 1976). Thus, contamination of the feces

by endogenous material could be related with the discrimination diet-feces (Martins et al. 2012). The fecal is composed mainly of bacterial and some endogenous matter (Van Soest, 1994). Sponheimer et al. (2003) found a negative fractionation between feces and herbivores diet, where feces had a greater proportion of acid detergent fiber, enriched in ^{13}C, compared with the diet. They expected a $\delta^{13}C$ value more positive, but assuming that after the use of acid detergent the microbiota was removed from feces, the $\delta^{13}C$ value should increase, therefore, the hypothesis of the influence of microbiota was not supported.

6. 3. 3 Different digestibility and $\delta^{13}C$ of animal diet

Different plant digestibility that are part of the animal diet can be related also with the $\delta^{13}C$ value between diet and feces (Jones et al., 1979). The $\delta^{13}C_{\text{diet-feces}}$ is influenced by the difference between the digestibility of the mixed diet, where the less digestible component is overestimated in the feces (Botha and Stock, 2005). Norman et al. (2009) classified the difference in the organic matter digestibility of the C_3 and C_4 components of the diet as one of the possible factors that contribute to errors in diet reconstruction, and the use of digestibility can improve the accuracy of the method. However, Wittmer et al. (2010) did not find influence of different digestibility of C_3 and C_4 species, for grazing animals. Thus, the different digestibility of sources did not affect the diet reconstruction from fecal $\delta^{13}C$ values (Sponheimer et al., 2003).

7. Discrimination of $\delta^{13}C$ in different plant tissue

In general, the analysis of $\delta^{13}C$ values are made on leaves, however, there are variation in $\delta^{13}C$ value among organs in the plants (O'leary, 1981). This variation is caused due to genetic and environment factors, linked with gas exchange by morphological and plant responses (Dawson et al., 2002). The tissues that are photosynthetic inefficient, such as stems and roots, are more enriched in ^{13}C than leaf tissue (O'Leary, 1988). In normal conditions, Ramírez et al.

(2015) found a $\delta^{13}C$ over 2‰ of leaves and tuber of three genotypes of potato. Differences among chemical components of plant tissue also influence the $\delta^{13}C$ of plants, as lignin that is 1- 2 ‰ lighter than the total plant (Marshall et al., 2007). Fernandez et al. (2003) observed that the lignin is depleted in ^{13}C compared to cellulose.

Difference in the $\delta^{13}C$ between lipid and the bulk material ranges from 5 to 10‰, being the lipid depleted in ^{13}C (O'leary, 1981). Ballentine et al. (1998) in a study with three C_4 plant genotypes found a discrimination between whole plant and lipid of sugar cane $\delta^{13}C$ of 5‰; in *Cenchrus ciliaris* the discrimination was 7‰ and in *Antephoras pubescence* it was 9‰. Sponheimer et al. (2003) evaluated the discrimination between plant material and acid-detergent fiber (ADF) in *Cynodon dactylon* and found -1.4 ‰, while alfalfa did not show significant difference. Thus, it is clear that different parts, compound and nutrients of a plant express a particular $\delta^{13}C$ value.

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Chapter 2

Tracing back ruminant diet feeding grass-legume mixtures using fecal δ ^{13}C

Resumo

Os isótopos estáveis podem ser uma importante ferramenta de pesquisa para rastrear C e N em experimentos de pastagem. Este estudo testou diferentes proporções de gramineas C_4 e leguminosa e a correlação entre $\delta^{13}C$ dietético com $\delta^{13}C$ fecal. Quarenta cordeiros, com peso corporal médio de 20,4 kg, foram casualizados em blocos e alimentados com feno de Tifton-85 (*Cynodon spp*.) e Alfafa (*Medicago sativa*), em diferentes níveis de substituição, compondo cinco tratamentos: 1) 100% de Tifton -85 feno; 2) 75% de Tifton-85 + 25% de feno de alfafa; 3) 50% de Tifton-85 + 50% de feno de alfafa; 4) 75% de alfafa + 25% de feno Tifton-85; 5) 100% feno de feno de alfafa. O experimento durou 27 dias, consistindo de 22 dias para a adaptação e 5 dias para a coleta de sobras, alimentos e fezes. As amostras fecais foram coletadas diretamente do reto, para evitar contaminação. Todas as amostras foram coletadas durante o período de amostragem de 5 dias, sendo composta no final. As amostras de alimentação e fezes foram secas por 72 horas em uma estufa (55ºC) e moídas em um moinho Willey em uma peneira de 2 mm, incubadas por 288 horas in situ para obter a indigestibilidade. Todas as amostras coletadas foram submetidas a fibra detergente neutro indigestível (FDNi), C, N e seus respectivos isótopos estáveis. O carbono total apresentou δ13C de -14,98, -18,22, -23,85, -25,99 e -30,64 ‰ para o tratamento de 1 a 5, respectivamente. O $\delta^{13}C$ de amostras tratadas com FDNi foi -17,41, -20,27, -26,06, -27,83 e -31,67 ‰, respectivamente. Fecal $\delta^{13}C$ para carbono total foi -16,23, -20,79, -25,10, -28,8 e -32,31 ‰, e para a amostra tratada com FDNi -16,65, -21,52, -26,25, 29,20 e -32,06 ‰ para o tratamento 1 a 5, respectivamente . As amostras de fezes foram mais negativas do que as amostras dietéticas, e o FDNi alterou o $\delta^{13}C$ fecal. Os modelos utilizados para calcular as dietas ajustadas para predizer a dieta por $\delta^{13}C$ fecal e os melhores modelos apresentaram R^2 de 0,98 usando amostras de carbono total, com resultados similares encontrados ao usar amostras de FDNi (R2 = 0,97). Houve uma discriminação ^{13}C entre amostras dietéticas e fecais, no entanto, a proporção de espécies C_3 e C_4 na dieta pode ser predita com precisão com base em amostras fecais usando $\delta^{13}C$. O uso de amostras tratadas com FDNi não melhorou os modelos, bem como a adição de digestibilidade e indigestibilidade, avaliada em cordeiros alimentados por diferentes níveis de alfafa e feno tifton-85.

Palavras-chave: discriminação, digestibilidade, back-calculation, carbono, FDNi

Abstract

Stable isotopes can be an important research tool to track C and N in grazing experiments. This study tested different proportions of C_4 grass and legume and the correlation between dietary $\delta^{13}C$ with fecal $\delta^{13}C$. Forty lambs, with average body weight of 20.4 kg, were randomized in a complete block design and fed Tifton-85 (*Cynodon spp.*) and Alfalfa (*Medicago sativa*) hays, at different levels of substitution composing five treatments: 1) 100% Tifton-85 hay; 2) 75% Tifton-85 + 25% alfalfa hay; 3) 50% Tifton-85 + 50% alfalfa hay; 4) 75% alfalfa + 25% Tifton-85 hay; 5) 100% hay of alfalfa hay. The trial lasted 27 days, consisting of 22 days for adaptation and 5 days for collection of orts, feed, and feces. Fecal samples were collected directly from the rectum, to avoid contamination. All samples were collected during the 5-d sampling period, being composited at the end. Feed and fecal samples were dried for 72 hours in an oven (55ºC) and ground in a Willey mill to pass a 2-mm sieve, incubated for 288 hours in situ to obtain the indigestibility. All collected samples were subjected to indigestible neutral detergent fiber (iNDF), C, N and their respective stable isotopes The total carbon had $\delta^{13}C$ of -14.98, -18.22, -23.85, -25.99, and -30.64‰ for treatment 1 to 5, respectively. The $\delta^{13}C$ of iNDF treated samples was -17.41, -20.27, -26.06, -27.83, and -31.67‰, respectively. Fecal $\delta^{13}C$ for total carbon was -16.23, -20.79, -25.10, -28.8, and -32.31‰, and for iNDF treated sample -16.65, -21.52, -26.25, 29.20, and -32.06‰ for treatment 1 to 5, respectively. Fecal samples were more depleted than dietary samples, and the iNDF changed the fecal $\delta^{13}C$. The models used to back calculate the diets fit to predict the diet by fecal $\delta^{13}C$, and the best models had R^2 of 0.98 using total carbon samples, with similar results found when using iNDF samples ($R^2 = 0.97$). There was a ^{13}C discrimination between dietary and fecal samples, however, the proportion of C_3 and C_4 species in the diet can be accurately predicted based on fecal samples using $\delta^{13}C$. The use of iNDF treated samples did not improve the models, as well as the addition of digestibility and indigestibility, evaluated in lambs fed by different levels of alfalfa and tifton-85 hay.

Key words: discrimination, digestibility, back-calculation, carbon, iNDF

Introduction

Grass and legume mixtures provide environmental, ecological and productive advantages. Legumes are not only a source of protein for livestock, but they also fix atmospheric-N_2, adding this element to grassland ecosystems (Dubeux et al. 2006). Nitrogen is one of the main factors limiting livestock productivity. Therefore, the addition of N-fixing legumes can increase primary productivity (Makkar, 2008). Including legumes to low quality grass diet, increases the digestibility and intake, which improve animal response (Muir et al., 2011). One major challenge, however, is to obtain a representative sample of what the animal is eating because of the animal selectivity. Moreover, a proxy for the real digestibility of the selected diet is difficult to obtain, although in many cases in vitro digestibility has been used (Carvalho et al., 2007).

When the pasture is a binary mixture of warm-season grass (C_4) and legume (C_3), these plants have two different ways to fix carbon. The physiological mechanism inherent to these distinct mechanism of fixation (C_3 vs. C_4), results in different levels of ^{13}C discrimination. Plants with C_3 physiology have a range of $\delta^{13}C$ from -26 to 32‰, and are more depleted in ^{13}C than plants with C_4 physiology, which range from -9 to -17‰, due to the difference in photosynthetic pathways (Ballentine et al., 1998; Fernandez et al., 2003).

Norman et al. (2009) reported that the natural abundance of stable isotopes and its variability among photosynthetic pathways can be used to estimate the proportion of C_3 and C_4 plants in ruminant diets. Macedo et al. (2010) could estimate the proportions of grasses and legumes in the diet of confined steers using fecal $\delta^{13}C$. Understanding diet composition of ruminants will permit to assess the individual contribution of the grass and the legume components on the animal performance. The relationship between fecal $\delta^{13}C$ and the diet of

907 grazing animals is a way to assess it. Martins et al. (2012) reported that female sheep fed on a
908 C_3 plant diet had different fecal samples than females fed on a C_4 diet.

909 Possible differences in forage digestibility and feed interactions in ruminal conditions
910 could drive to wrong conclusions in a direct correlation of fecal $\delta^{13}C$ with diet composition.
911 Feces and diet are often different in $\delta^{13}C$ value. Some of the possible factors are endogenous
912 source of C, possible discrimination in the gut via microorganisms, and differences in
913 digestibility between the C_3 and C_4 species (Jones et al., 1979; Martins et al., 2012). The use
914 of neutral detergent fiber (NDF) analysis, which represent the fraction, that is unavailable for
915 microbial digestion in ruminant (Raffrenato et al., 2013), can be a useful tool to correct the
916 differences of $\delta^{13}C$ from feces to diet. This process could eliminate endogenous and microbial
917 contamination present in the feces.

918 A feedlot trial in controlled conditions where intake and excreta are monitored is the best
919 option to validate models. These models could ultimately be applied to grazing animals, in
920 order to evaluate the proportions of C_3 and C_4 in animal diets. Thus, the objective of this
921 research was to assess the proportion of C_3 and C_4 forage species in the diet using iNDF $\delta^{13}C$,
922 based on fecal and dietary samples. We hypothesized that by analyzing the $\delta^{13}C$ on the iNDF
923 we could estimate more accurately the proportion of the different species in the diet, reducing
924 contamination with endogenous carbon.

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Material and Methods

Experimental site

The experiment was conducted at the Federal Rural University of Pernambuco (UFRPE), in the small ruminant sector of the Animal Science Department located in Recife (8°01"S, 34°57'W,14 m asl), Pernambuco, Brazil. The temperature during the experimental period varied between 22 and 31°C (APAC, 2017).

Animals and experimental diets

Experimental animals were forty lambs, Santa Ines mixed breed, with an average age of 3 months and body weight of 20.4 kg ± 1.8. Animals were arranged in a randomized complete block design and placed in individual pens. The initial weight was used as a blocking criterion. Before the beginning of the trial, anthelminthic was administered to all animals, and then they were labeled, and randomly housed in pens within each block, with access to water and feeding troughs.

Experimental diets (5) were replacement levels of Tifton-85 (*Cynodon spp.*) and Alfalfa (*Medicago sativa*) hay: 1) 100% Tifton-85 hay; 2) 75% Tifton-85 + 25% alfalfa hay; 3) 50% Tifton-85 + 50% alfalfa hay; 4) 75% alfalfa + 25% Tifton-85 hay; 5) 100% alfalfa hay. Diet chemical composition is presented on Table 1. The alfalfa and Tifton-85 hay were obtained from a local producer. The hay was ground in a forage grinder (Laboremus, Campina Grande, PB, BR) to pass a 8-mm sieve with the objective of reducing the particle size in order to improve the mixture of the material and avoid animal selectivity. Feed supply was adjusted to 3.4% of the body weight on a dry matter basis, reducing orts. Intake of the whole diet in each meal was recorded. Water was offered *ad libitum*, being replaced every two days. Sheep were fed twice daily, at 8:00 am and 3:00 pm. The experiment lasted 27 days, from December 11th

954 of 2015 to January 8th, 2016, consisting of 22 days of adaptation and the five last days for
955 sample collection.

Table 1. Chemical composition of different levels of alfalfa and tifton 85 hay

	Tifton-85: Alfalfa				
	0:100	25:75	50:50	75:25	100:0
DM g kg^{-1}	915	910	913	910	907
CP g kg^{-1}	98	99	112	128	131
NDF g kg^{-1}	810	765	652	680	548
iNDF g kg^{-1}	255	230	252	298	340

956 DM = dry matter; CP = crude protein; NDF = neutral detergent fiber; iNDF = indigestible neutral detergent fiber
957
958 *Sample collection*

959 Fecal samples were collected directly from the rectum, to avoid contamination (Martins
960 et al., 2012). Samples were collected twice daily, at different times, during five consecutive
961 days: 8 am, 1 pm; 9 am, 2 pm; 10 am, 3 pm; 11 am, 4 pm; and 12 pm, 5 pm. Orts were collected
962 before the morning feeding, during the collection period. All samples were weighed, dried for
963 72 hours in a forced air circulation oven at 55°C, and then ground in a Willey mill (Tecnal®,
964 Piracicaba, SP, BR) to pass a 2-mm sieve.

965 *Digestibility and carbon stable isotopic analyses*

966 Total fecal DM output was determined using iNDF as an internal marker. Samples of
967 diets, orts, and feces were weighed in an analytic balance (1.0 g precision), and placed into
968 TNT (nonwoven fabric; polypropylene) bags measuring 5 x 5 cm, and porosity of 100 µm.
969 Bags were replicated twice and incubated in situ for 288 h in a rumen fistulated dairy cow, with
970 575 kg body weight, fed with a diet based on 80:20 (hay:concentrate) according to Detmann et
971 al. (2012). After the incubation period, the bags were thoroughly washed in fresh water and
972 dried at 55°C for 72 hours, and then oven-dried for 105°C, for 45 minutes, following instruction

of (Casali et al., 2008). The material incubated was submitted to NDF analysis (Detmann et al., 2012), and the iNDF samples were used to analyze the $\delta^{13}C$.

Feed intake was obtained by the difference between feed offered and orts. Diet DM digestibility was determined using the difference between feed intake and fecal output, divided by the feed intake (Azevedo et al., 2014):

$$A) \quad Digestibility\ of\ Diet\ DM = \frac{Intake\ - Total\ Fecal\ Output}{Intake}$$

Stable isotopic analyses were performed at the Forage Laboratory from the University of Florida - North Florida Research and Education Center (NFREC), located in Marianna, Florida. All samples and material remaining from the iNDF process were ball milled to reduce the particle size under 100 µm, before the stable isotope analyses (Michener and Lajtha, 2007). Samples were ball milled using a Mixer Mill MM400 (Retsch, Newton, PA, USA) at 25 Hz for 9 min. Samples were analyzed for total C and N using a CHNS analyzer through the Dumas dry combustion method (Vario Micro Cube; Elementar, Hanau, GER) coupled to an isotope ratio mass spectrometer (IsoPrime 100, IsoPrime, Manchester, UK) to analyze $\delta^{13}C$ and $\delta^{15}N$. The $^{13}C/^{12}C$ ratios are presented in the conventional delta (δ) notation, in per mil (‰) relative to the Pee Dee Belemnite (PBD). Crude protein was estimated multiplying total N concentration by 6.25.

Equation B, used to find the notation $\delta^{13}C$:

$$B) \quad \delta^{M}E = \left[\left({R_{Sample}}/{R_{Standard}}\right) - 1\right] * 1000$$

Where E denotes the element, M is the mass of the heavy isotope, R is the ratio of the heavy to light isotope for E, thus, the ratio of $^{13}C:^{12}C$ is expressed as $\delta^{13}C$ (Ehleringer & Rundel, 1989; Dawson & Brooks, 2001; Crawford et al., 2008).

Plant C sources from fecal samples were identified by the equation C, two-mixing pool, (Fry, 2006):

$$C) \qquad f1 = \frac{\delta\ sample - \delta\ source\ 2}{\delta\ source 1 - \ \delta\ source\ 2}$$

$$f1 + f2 = 1\ (or\ f1 = 1 - f2\ )$$

Where f 1 represents the fraction (contribuition) of source 1 e f 2 of source 2

The same equation above was used to determine the real proportion of C_3 and C_4 forage ingested by the sheep. The equation was applied to obtain the proportion (and amount) of each one of these diet components in the orts, for each individual animal, that were then subtracted from the supplied amount in order to find the real intake of each component.

In order to estimate the proportion of each source in the diet, we also tested four models described by Norman et al. (2009) to back-calculate the diet. All the equations determined the proportion of C_4 in the diet using fecal samples values. These equations were tested using $\delta^{13}C$ determined on total C (sample without passing through the iNDF procedure) or in samples after passing through the iNDF procedure. The equation 1 is the same equation described above by Fry (2006) to mixed sources of C.

1. $\%C4 = 100 - \{100 * [(A - C)/(B - C)]\}$

Where

$\%C_4$ = Percentage of forage intake based on DM and in this case, a C_4 grass
A = $\delta^{13}C$ of the animal feces
B = $\delta^{13}C$ of the C_3 Plant
C = $\delta^{13}C$ of the C_4 Plant

Eq. (2) is similar to eq. (1) but the discrimination between diet and feces was included. According to Norman et al. (2009), this equation assumes that diet-tissue discrimination rates are the same for both the C_3 and C_4 component of the diet.

$$2.\quad \%C4 = 100 - \{100 * [(A - C - J)/(B - C)]\}$$

J = diet – feces discrimination*

*Diet – Feces discrimination = δ^{13}C of the animal feces - δ^{13}C of the diet

This study used the discrimination as a constant number. The value used was the average of all discriminations, between all the treatments. The J value was -1.63‰ and it was obtained from the current dataset for this trial.

Two different equations were used to assess the effect of *in vivo* indigestibility and digestibility. Norman et al. (2006) reported that for feces, the back calculation should correct for relative indigestibility (eq. 3a), and the samples as blood or wool should be corrected with relative digestibility. This study just used feces to back calculate the diet, then eq. 4 will represent only samples of feces.

$$3a.\quad \%C4 = 100 - 100/(\{[F * ((B + J) - A)]/[G * (A - (C + J)))\ ]\} + 1)$$

$$3b.\quad \%C4 = 100 - 100/(\{[D * ((B + J) - A)]/[E * (A - (C + J)))\ ]\} + 1)$$

Where

D = % Dry matter *in vivo* digestibility of C_3 plants
E = % Dry matter *in vivo* digestibility of C_4 plants
F = 100 - % Dry matter *in vivo* digestibility of C_3 plants
G = 100 - % Dry matter *in vivo* digestibility of C_4 plants

Eq. (4) uses the δ^{13}C of the animal feces and the δ^{13}C of feces collected from animals that ate exclusively plants of C_3 or C_4, in order to back calculate the proportion in the diet.

4. $\%C4 = 100 - \{100 * [(A - I)/(H - I)]\}$

Where

H = $\delta^{13}C$ of the same type of sample from animal on 100% C_3 diet
I = $\delta^{13}C$ of the same type of sample from animal on 100% C_4 diet

Statistical analyses

Data were analyzed using PROC MIXED of SAS (SAS Inst. Inc., Cary, NC, USA). Fixed effects included treatments. Blocks were considered random effect. LSMEANS were compared using the PDIFF procedure from SAS adjusted by Tukey ($P < 0.05$). Regression equations between the measured proportion and estimated proportion, for each one of the tested models, were performed using PROC REG of SAS.

Results and Discussion

Dry matter intake and total fecal output

Table 2. Total intake, legume and grass intake, and total fecal output of lamb fed different levels of alfalfa and Tifton-85 hay

	Intake %BW	Dry matter intake (g DM head^{-1} day^{-1})	Grass intake† (g DM head^{-1} day^{-1})	Legume intake† (g DM head^{-1} day^{-1})	Total fecal output (g DM head^{-1} day^{-1})
Tifton-85:Alfalfa*					
100:0	2.19	480	470	---	250
75:25	2.65	590	420	150	330
50:50	2.65	610	290	320	320
25:75	3.03	690	170	510	390
0:100	3.11	750	---	750	470
L (P value)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Q (P value)	N. S	N. S	0.0150	0.0150	N. S

L = Linear effect; Q = Quadratic effect; N.S = No significant.
* Target proportion, values do not include orts.
† Values obtained by orts

There was a linear increase (P<0.0001) in dry matter intake (DMI) and in the intake as a percentage of body weight (BW) with increasing levels of alfalfa hay in the diet (Table 2). Grass DMI on 100% grass diet was 470 g DM hd^{-1} d^{-1}, while the pure legume DMI was 750 g DM hd^{-1} d^{-1}. There was a quadratic effect (P= 0.0150) for legume and grass intake. The obtained intake increment due to the replacement of grass by legume was expected and it was equivalent to that reported by Burns and Fisher (2013) replacing the diet of steers from mature grass to alfalfa, at different levels. In this present study, Tifton-85 hay had greater NDF concentration (Table 1). Thus, the replacement of grass by legume decreased the NDF concentration in the diet, increasing DMI as a result. Van Soest (1994) reported that low forage quality and high fiber concentration in the diet limit forage intake. However, chemical degradation and physical breakdown in the rumen directly influence legume intake in relation to grass, because of the rumen filling effect (Dewhurst et al., 2009). Low-energy and high-filling diets depress intake by a physical mechanism. Thereby, NDF is considered a feed characteristic that is directly related to the filling effect of the diet (Mertens, 2007). NDF digestibility also plays a role, considering that different compounds such as lignin, cellulose, and hemicellulose can make up different proportions of the NDF, depending on the forage species, environmental conditions, and maturity of the plant (Van Soest, 1994).

Alfalfa hay DMI in the pure diet was similar to the intake values (67 to 74 g kg d^{-1} $BW^{0.75}$) observed by Wildeus et al. (2007) for three breeds of wool sheep. This result is also consistent with Park et al. (1989) who found greater DMI for alfalfa than for bermudagrass (coastal), 61.3 g kg d^{-1} $BW^{0.75}$ and 57.8 g kg d^{-1} $BW^{0.75}$ respectively. In addition to the preference for alfalfa hay, grass fiber concentration might have contributed to the reduced intake. In this study, Tifton-85 hay NDF concentration (810 g kg^{-1}) was greater than the NDF concentration found by Lopes (2015; 756.1 g kg^{-1} DM) and Coelho (2015; 703 g kg DM) for the same grass. Increase of intake after inclusion of alfalfa in the diet is also supported by

Manaye et al. (2009), who observed an increase of DMI from 57.9 g kg $BW^{-0.75}$ to 63.1 g kg $BW^{-0.75}$ after the supplementation of legume in the grass-based diet of sheep.

Grass NDF in that study (715 g kg^{-1} DM) was greater than legume NDF (399 g kg^{-1} DM). Tamir and Asefa (2009) studied the effect of legume inclusion (*Acacia saligna*) on the voluntary feed intake of lambs on a diet based on grass hay. Legume inclusion improved total DMI of lambs. Przemysław et al. (2015) reported greater DMI of alfalfa (cv. Alba) silage, than red clover (cv. Nike), and red fescue (cv. Godolin) silage to lambs. The NDF concentrations of red clover and fescue were 509 and 590 g kg^{-1} DM, respectively, greater than the NDF concentration of alfalfa silage, which was 462 g kg^{-1} DM, resulting in a lower DMI. Bamikole et al. (2001) observed a different result when feeding goat with grass–legume mixture, and fertilized and unfertilized grass. The DMI was considered similar among these diets (208, 244, and 220 g kg^{-1} DM d^{-1}, respectively) with equivalent NDF concentration.

Particle size is another factor that might have contributed to increase forage intake. Smaller feed particles increase passage rate and feed intake, but reduces digestibility. Van Soest (1994) indicated that the reduction of particle size before feeding, by grinding, can increase forage intake. Smaller feed particles increase diet density, which leads to faster passage rates and less rumen volume, reducing rumination time as a result. Particle size reduction is crucial for disruption of the cuticle and cell wall membranes, exposing cell contents and enhancing access for microbes and enzymes (Mertens, 2007). Legumes, in general, have less NDF concentration than warm-season grasses, being more easily degradable by rumen microorganism.

The alfalfa and Tifton-85 hay used in this study were ground to improve intake, reduce selectivity, and minimize orts. The amount of forage offered was limited to 3% of the BW; limiting the intake and reducing the selectivity of plant parts, as well as of forage species. The

1110 homogenization of the mixed diet was important to avoid variation. When diet components are
1111 not thoroughly mixed, animals tend to select the most digestible components. Because of
1112 animal preference and orts, the real intake is different from the supplied diet (Mertens, 2007).

1113 Total fecal output (TFO) increased linearly ($P < 0.0001$) with the inclusion of legume
1114 in the diet. The TFO of animals that ate pure grass was 0.25 kg DM head^{-1} d^{-1} and increased
1115 with increasing levels of legume in the diet up to 0.47 kg DM head^{-1} d^{-1} for the animals fed on
1116 pure legume diet.

1117 *Total Apparent digestibility*

1118

Table 3. Total apparent digestibility, and alfalfa and tifton-85 apparent digestibility on lamb fed by different levels of alfalfa and Tifton-85 hay

Tifton-85: Alfalfa	Mixtures (g kg^{-1})	Tifton-85 (g kg^{-1})	Alfalfa (g kg^{-1})
100:0	---	473	---
75:25	423	441	367
50:50	479	487	470
25:75	430	494	409
0:100	---	---	374
Q (P value)	0.0149	< 0.0001	< 0.0001

Q = Quadratic effect

1119 Total apparent digestibility of the mixed diets had a quadratic ($P = 0.0149$) effect among
1120 diet levels (Table 3). The apparent digestibility of grass and legume in pure diets were 473 g
1121 kg^{-1} and 374 g kg^{-1}, respectively. The 75:25 mixture of Tifton-85 and alfalfa was 423 g kg^{-1},
1122 the lowest digestibility among the mixtures. Increasing the proportion of alfalfa to 50%
1123 improved the digestibility of the mixed diet from 423 g kg^{-1} to 479 g kg^{-1}. Replacing Tifton-85
1124 by alfalfa from 50% to 75%, however, reduced the digestibility of the mixed diet to 430 g kg^{-}
1125 ^{1}.

There was a quadratic effect (P <0.0001) for the digestibility of the individual components in the mixed diets, varying with their participation in each mixed diet. The mixture of Tifton-85 and alfalfa up to 50% improved both digestibilities. Alfalfa digestibility increased from 374 to 470 g kg^{-1}, and Tifton-85 from 473 to 487 g kg^{-1}, for 100% to 50% inclusion, respectively. Increasing levels of alfalfa improved Tifton-85 digestibility.

Alfalfa digestibility was untypically low, and differed from literature values. Parker et al. (1989) observed alfalfa digestibility of 673 g kg^{-1}, Wildeus et al. (2007) reported alfalfa digestibility ranging from 583 to 630 g kg^{-1}, and Burns and Fisher (2013) as 788 g kg^{-1}. This unusual result was probably due to the reduced particle size of the alfalfa hay, maturity stage, and iNDF concentration. The same hay was incubated (*in situ*) in a ruminal fistulated cow and the digestibility was 570 g kg^{-1} DM (Table 3). This value is similar to that obtained by Nelson and Satter (1992), who found 560 g kg^{-1} DM in late cut alfalfa hay and greater than 516 g kg^{-1} DM of alfalfa at early flower maturity stage after 36 hours of incubation, obtained by Yari et al. (2012). Alfalfa digestibility from the *in vivo* trial, however, is lower than the one obtained from the *in situ* digestibility trial. The time of 48 h used to determine the digestibility *in situ*, could, contribute to this value, due to the opportunity to degrade. Furthermore, the passage rate was not an issue in this case, as observed in the *in vivo* trial.

The mature alfalfa studied by Yari et al. (2012) had CP of 162 g kg^{-1} DM and NDF of 491 g kg^{-1} DM, values different than the ones obtained in this study, i.e. 131 g kg^{-1} DM for CP and 548 g kg^{-1} DM and NDF (Table 1). The alfalfa used in this study was poor in protein and high in fiber, reasons that can be related to its low digestibility. Lacefield et al. (2009) reported values for alfalfa CP ranging from 90 to 130 g kg^{-1} DM and NDF from 560 to 600 g kg^{-1} DM at late bloom maturity stage. These values are similar to the ones obtained in this study. Palmonari et al. (2014) reported that alfalfa maturity influences its CP and fiber, affecting significantly the digestibility. Later maturity is responsible for greater fiber and lignin

concentrations and reduced CP, digestibility and alfalfa hay quality (Lacefield et al., 2009; Yari et al. 2014).

The alfalfa and Tifton-85 hay were ground in a forage grinder to facilitate the homogenization of the mixtures. The sieve used to grind was 8 mm, which possibly reduced the particle size down to a point that affected passage rate and digestibility (Mertens, 2007). Van Soest (1994) can also explain the low digestibility observed for the alfalfa used in this study. Finer feed particle degrades faster due to increased surface area. Reduced time for ruminal degradation, however, results in less rumination, because of the particle size density, resulting in lower digestibility. The grinding effect can be compared to rumination in terms of digestive effect (Minson, 1990). For sheep, the consequence of hay small particles is the reduction of the time in the tract, reducing the digestibility of the organic matter and fiber.

The iNDF concentration represents the indigestible fraction of the forage and can be considered another factor responsible to reduce digestibility. The alfalfa hay used in this study had 330 g kg^{-1} DM of iNDF (Table 1) and differed from the iNDF of mature alfalfa analyzed by Palmonari et al. (2014) in a two-year study, that was 167 g kg^{-1} DM and 175 g kg^{-1} DM.

Dietary and fecal $\delta^{13}C$ based on total C and iNDF samples

As expected, increasing levels of alfalfa resulted in more depleted samples in ^{13}C, both for fecal and dietary samples (Table 4). All fecal samples were more depleted than diet samples, except the iNDF-treated samples in which 50% of each component was supplied. Average discrimination between feces and diet was greater for untreated samples than for iNDF-treated samples.

Table 4. Dietary and fecal $\delta^{13}C$, and discrimination based on total sample C and indigestible neutral fiber treated samples

Tifton-85: Alfalfa	Diet (‰)	Feces (‰)	Δδ	*P* value
		Total C samples		
100:0	-14.98	-16.23	-1.25	<0.0001
75:25	-18.22	-20.79	-2.57	<0.0001
50:50	-23.85	-25.10	-1.25	0.00091
25:75	-25.99	-28.78	-2.75	<0.0001
0:100	-30.64	-32.31	-1.67	<0.0001
		iNDF treated samples		
100:0	-17.41	-16.56	0.85	<0.0001
75:25	-20.27	-21.52	-1.25	<0.0001
50:50	-26.09	-26.25	-0.17	0.40480
25:75	-27.83	-29.20	-1.37	0.00071
0:100	-31.67	-32.06	-0.39	0.00332

P value = T Student ($p>0.05$) comparing $\delta^{13}C$ between diet and feces samples; Δδ = difference between feces and diet

Grass diet $\delta^{13}C$ was -14.98‰ and fecal $\delta^{13}C$ was -16.23‰, with a discrimination of –1.25‰, while legume diet $\delta^{13}C$ was -30.64‰ and fecal $\delta^{13}C$ was -32.31‰, with a discrimination of -1.67‰. The $\delta^{13}C$ assessed from feces in this present study was more depleted than the diet $\delta^{13}C$. The result obtained in the pure legume diet differ with the reported by Sponheimer et al. (2003), Macedo et al. (2010), and Martins et al. (2012), where they did not identify $\delta^{13}C$ difference between feces and legume diet. The discrimination between diet and feces, in total carbon samples observed in this study, were in the same range observed by Jones et al. (1979); Sponheimer et al. (2003); Hwang et al. (2007); Norman et al. (2009); and Macedo et al. (2010).

Diet and fecal $\delta^{13}C$ assessed on iNDF-treated samples indicated that these samples became more depleted after iNDF analysis. Pure diets and feces had $\delta^{13}C$ of -17.41 and -16.56‰ ($P>0.0001$) for grass and -31.67‰ and -32.06‰ ($P=0.00322$) for legume, respectively. The Δδ for grass and legume were 0.85 and -0.39‰, respectively. For mixed diets,

the $\delta^{13}C$ value became depleted when legumes were included in the mixture, as observed with samples based on total carbon. In the diet with 25% of legume inclusion, the $\delta^{13}C$ was -20.27‰ and the feces, -21.52‰ (*P<0.0001*). When the amounts of legume and grass were the same (50:50), the $\delta^{13}C$ value of the diet was -26.09‰ and the feces -26.25‰ and the $\Delta\delta$ of – 0.17 ‰ was not significantly different (*P=0.4048*). The $\delta^{13}C$ values of diet and feces, with 75% of legume inclusion, were -27.83‰ and -29.20‰ (*P=0.0070*), respectively, with a $\Delta\delta$ value of -1.37‰.

The $\delta^{13}C$ results for alfalfa and Tifton-85 were within the range of C_3 and C_4 plants (Ballentine et al.,1998), alfalfa being more depleted than the one used by Sponheimer et al. (2003), which was -27.0‰. Martins et al. (2012) found $\delta^{13}C$ -31.15‰ in alfalfa hay, with NDF concentration of 574.5 g kg^{-1} DM, values similar to the ones found in this study (NDF = 548 g kg^{-1} DM). Alfalfa hay $\delta^{13}C$ in the present study was more depleted, perhaps, due to greater NDF concentration, because of the maturity stage of the plant. Leaf and stem ratio can influence the fractionation of $\delta^{13}C$ in the bulk analysis. Leaves are more enriched in $\delta^{13}C$ than stems and roots (O`Leary, 1988). Stems function as plant support and are rich in fiber and lignin (Taiz and Zeiger, 2002), differently from leaves, that are responsible for photosynthesis. Fernandez et al. (2003) reported that the $\delta^{13}C$ value of lignin, cellulose, and the bulk of *Lolium perene*, were -30.3‰, -26.50‰, and -28.5‰, respectively. Greater proportion of stem, with greater fiber and lignin levels, can lead to more negative $\delta^{13}C$ values.

In addition, the temperature of the region where these plants grew possibly pushed down the $\delta^{13}C$ value. O`Leary (1988) reported that environmental effects on $\delta^{13}C$ value in C_3 plants can occur due to the increase in temperature, which can move the signal to 3‰, negative. Thus, the signal of plants of the same species, and the same metabolism, can vary (Farquhar et al.,1982).

Tifton-85 hay $\delta^{13}C$ was compatible with the ones observed for C_4 plants. The value found by the present study was similar to the ones observed by Sponheimer et al. (2003), who reported -14.1‰ for bermudagrass (*Cynodon dactylon*) and by Norman et al. (2009) that found -14.9‰, for saltbush (*Atriplex nummularia Lindl.*).

The $\delta^{13}C$ value of grass and legume, from iNDF-treated samples,were also similar to the ones reported for the C_3 and C_4 plants (Ballentine et al.,1998). The $\delta^{13}C$ on treated samples, however, changed after the incubation, certainly, due to ruminal degradation and NDF analysis. The $\delta^{13}C$ of diets that were submited to the iNDF process became more depleted, and the feces stayed in the same pattern than the total carbon samples. However, the grass diet had a different discrimination, feces becaming more positive than diet, a situation different from the rest of the diets. The diet became more negative than the diets before incubation, as the rest of the diets. Probably, this discrimination came from the effect of the incubation plus the NDF solution, in this grass. Samples from this diet were degraded by microorganisms from rumen during 288 h, and were washed in neutral detergent thereafter, which removed some parts of the fiber; the same happened in the incubation. The signal obtained was different than those obtained in the feces, so the negative signature indicates greater proportion of indigestible fibers in this sample. The diet with 50% of grass and 50% of legume, after iNDF treatment, had the same signal as the feces.

The feces after iNDF process became -0.85‰, more negative than feces based on total carbon. This likely occurred because NDF incubation and NDF solution might have changed the signal of the diet, becoming similar to the ones observed in the feces. Discrimination between feces and diet is sometimes reported in the literature as an influence of endogenous contamination of the feces from gut tissue, microorganisms and other sources of carbon (Jones et al., 1979; Norman et al., 2009; Martins et al., 2012). This is not clear in the results presented in this study, since the NDF solution changed the signal of the diet, which became more

negative, but did not affect the fecal $\delta^{13}C$. The contaminations present in the feces must have been removed after the NDF process, altering the $\delta^{13}C$. Sponheimer et al. (2003) observed that after treating samples using the ADF process, the fecal $\delta^{13}C$ did not change. They related that the contamination by microorganism could not be the factor that turn the fecal $\delta^{13}C$ negative, because the effect of ADF solution, that can remove it.

One possible reason for this discrimination between diet and feces is the degradation of the plant material by rumen microorganisms. Fractionation of plant components is different, varying the $\delta^{13}C$ of protein, carbohydrate, lipids, fiber, and lignin (Ballentaine et al 1998; Fernandez et al. 2003). The action of microbes over the vegetal tissue removes components from the feces, that have different $\delta^{13}C$, and they may be depleted or enriched. The iNDF process showed that samples incubated in the bags for 12 days will represent the indigestible fraction, fiber that was not degraded by the bacteria, and has a different $\delta^{13}C$ free of contamination. Sponheimer et al. (2003) demonstrated that alfalfa has the same bulk and ADF signature, -27.0 and -27.1‰ respectively, and bermudagrass a bulk more negative than ADF, -14.1 and -12.7‰. Fernandez et al. (2003) reported that lignin of corn is depleted in ^{13}C, compared to cellulose, and bulk material.

The iNDF process did not reduce the discrimination between diet and feces. The iNDF changed the signature of diets, and had no effect in feces. Lignin was more depleted than the whole plant. The material that remained after the iNDF procedure is mostly represented, by lignin, and cellulose. In mixed diets, the lignin of legume and grass are different in $\delta^{13}C$, thus, in feces, the signal is going to be the $\delta^{13}C$ of lignin and cellulose, and cannot be reversed, by iNDF of feces, used to correct the fecal signal.

Models used to back calculate the animal diet

All models used to predict the proportion of C4 plants in the diet, total carbon and iNDF residue, fitted to predicted values ($P < 0.0001$). Equations 1, 2, and 4 had the same adjusted R^2, coefficient of variation, and root mean square error in total carbon samples and iNDF-treated samples. Equations 3a and 3b had the lowest adjusted R^2 among all equations in the total carbon analysis, as well as the greater CV and RMSE (Table 5). For iNDF-treated samples, the pattern was similar, with equation 3a having the lowest RMSE and CV and the greatest adjusted R^2 (Table 5). The models that used the total carbon to predict the C_4 proportions are presented in the Figure 1, and the models that used iNDF residue are presented in the Figure 2.

Table 5. Models using total sample carbon and indigestible neutral fiber $\delta^{13}C$ to trace back the diet of ruminants

Total C samples			
Models	Adjusted R^2	CV (%)	RMSE
1	0.986	8.5	4.2
2	0.986	8.5	4.2
3a	0.978	11.0	5.3
3b	0.975	11.9	5.8
4	0.986	8.5	4.2
iNDF-treated samples			
1	0.973	11.7	5.8
2	0.973	11.7	5.8
3a	0.979	10.7	5.2
3b	0.954	16.1	7.8
4	0.974	11.7	5.8

CV = coefficient of variation; RMSE = Root mean square error

Eq 1: $\%C_4=100- \{100*[(\delta^{13}C \text{ feces} - \delta^{13}C; C_4 \text{ plants})/ (\delta^{13}C; C_3 \text{ plants} - \delta^{13}C; C_4 \text{ plants})]\}$

Eq 2: $\%C_4=100- \{100*[(\delta^{13}C \text{ feces} - \delta^{13}C; C_4 \text{ plants}-\Delta\delta)/ (\delta^{13}C; C_3 \text{ plants} - \delta^{13}C; C_4 \text{ plants})]\}$

Eq 3a: $\%C_4=100-100/ (\{[\text{Indigestibility } C_3*((\delta^{13}C; C_3 \text{ plants} + \Delta\delta)- \delta^{13}C \text{ feces})]/ [\text{ Indigestibility } C_4*(\delta^{13}C \text{ feces} -(\delta^{13}C; C_4 \text{ plants} +\Delta\delta)))]\} +1)$

Eq 3b: $\%C_4=100-100/ (\{[\text{ Digestibility } C_3*((\delta^{13}C; C_3 \text{ plants} + \Delta\delta)- \delta^{13}C \text{ feces})]/ [\text{ Digestibility } C_4*(\delta^{13}C \text{ feces} -(\delta^{13}C; C_4 \text{ plants} +\Delta\delta)))]\} +1)$

Eq 4: $\%C_4=100- \{100*[(\delta^{13}C \text{ feces} - \delta^{13}C \text{ feces}; C_3)/ (\delta^{13}C \text{ feces}; C_4- \delta^{13}C \text{ feces}; C_3)]\}$

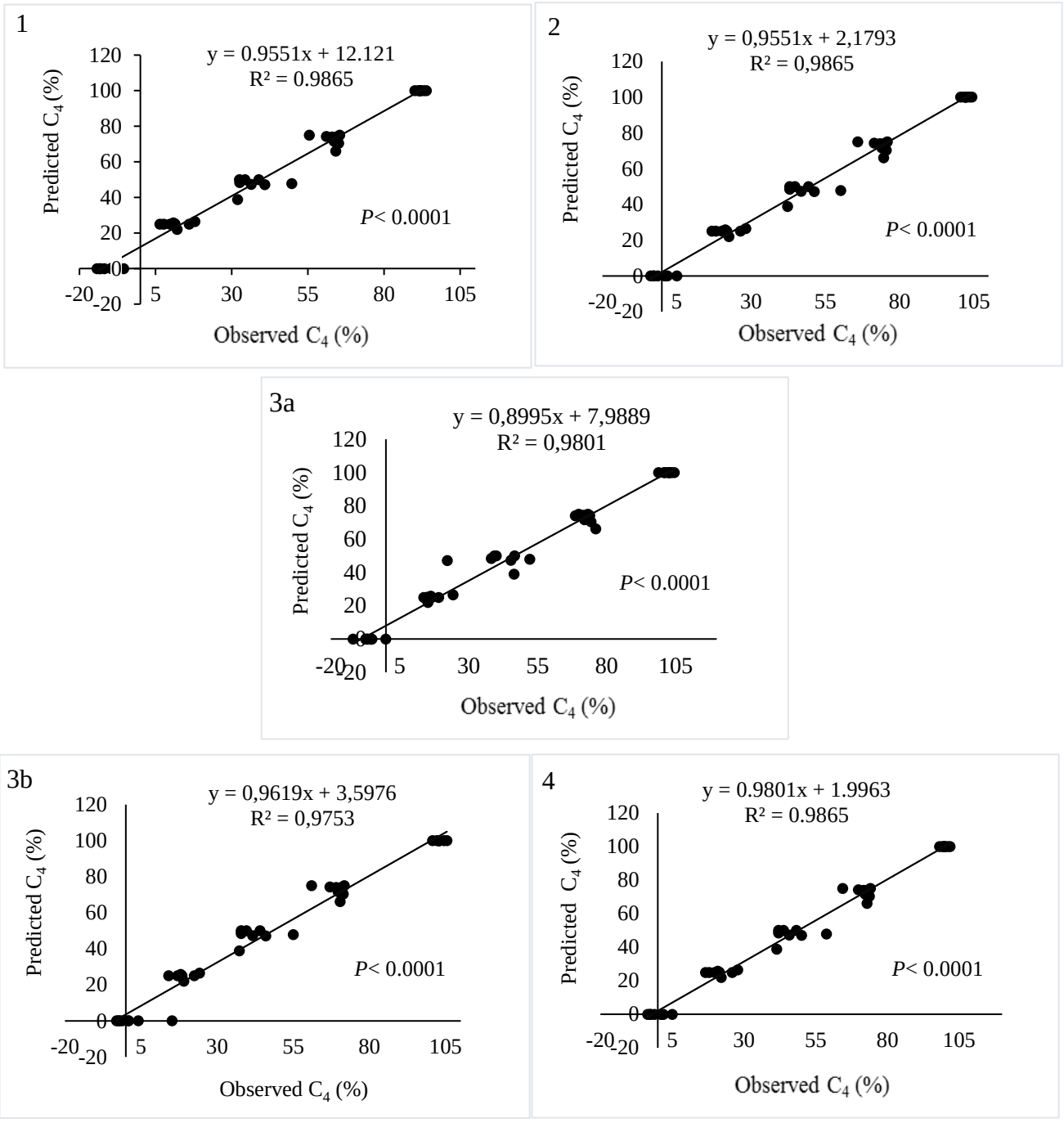

Figure 1. Proportion of C_4 plant in the diet predicted by models, using total carbon values. 1= % C_4 vs equation 1; 2 = % C_4 vs equation 2; 3a = % C_4 vs equation 3a; 3b = % C_4 vs equation 3b; 4 = % C_4 vs equation 4.

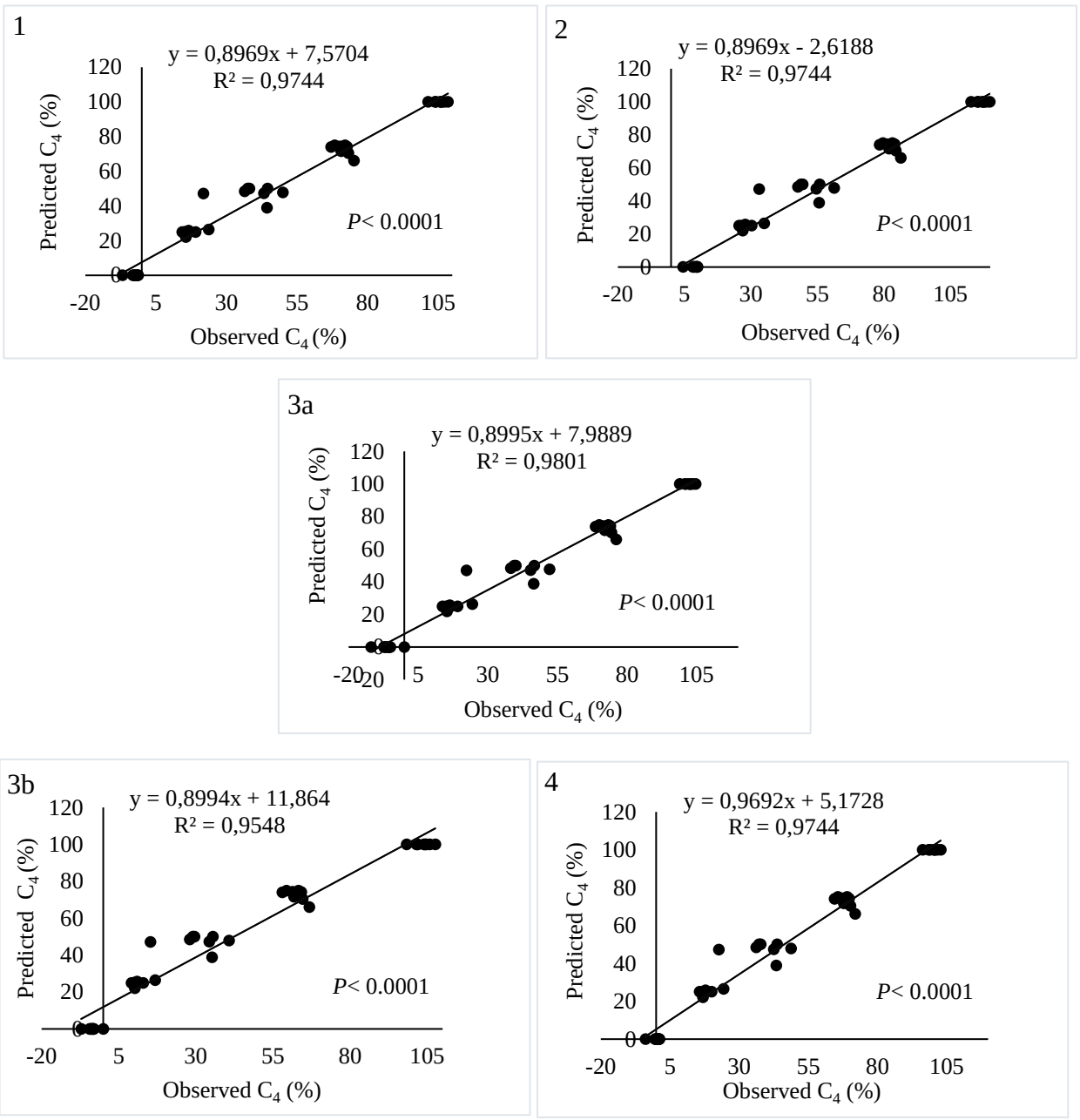

Figure 2. Proportion of C_4 plant in the diet predicted by models, using iNDF values. 1= % C_4 vs equation 1; 2 = % C_4 vs equation 2; 3a = % C_4 vs equation 3a; 3b = % C_4 vs equation 3b; 4 = % C_4 vs equation 4.

All the models from Norman et al. (2009) were suitable to predict the diet of the animals based on fecal sample $\delta^{13}C$. Equations 1, 2, and 4 are similar, and have the best fitness among the models. Norman et al. (2009) observed this pattern as well, with eq. 1, 2, and 4 presenting the same R^2 = 0.979. Equation 1 is a basic model, equation 2 is the addition of the

discrimination, and the equation 4 requires the separate discrimination of C_3 and C_4 plant in diet and feces. These equations are related with the addition of a constant number, therefore, the data are similar. Equation 3a and 3b are similar to equation 2, but with addition of indigestibility or digestibility and the addition of this component increased the error. The addition of the indigestibility did not improve the accuracy, differently from the data obtained by Norman et al. (2009), which the R^2 was 0.994. In general, the methods to predict are suitable, but, for grazing conditions, equation 1 is more practical because it has less components than the other equations.

Our hypothesis that the iNDF residue can improve the accuracy of the diet prediction, is not supported by these results. The results presented can clarify that, the use of $\delta^{13}C$ from iNDF components did not increase the prediction. The iNDF process changed the $\delta^{13}C$ of the diet. These results confirm these obtained by Jones et al. (1979), when they used fecal NDF in sheep feces and did not improve the relationship between diet and feces.

Conclusion

There is a ^{13}C discrimination between dietary and fecal samples. However, the proportion of C_3 and C_4 species in the diet can be accurately predicted based on fecal samples using $\delta^{13}C$. The addition of digestibility and indigestibility did not improve the models in this study. The use of iNDF did not improve the prediction compared with the total sample. All the models fitted to predict, however, the equation 4 has one factor that can reduce its use, which is the inclusion of $\delta^{13}C$ from feces of animals on exclusive C_3 plants diet. Equation 1 is recommended to back calculate the proportion of C_3 and C_4 plants in diets of lambs based on fecal $\delta^{13}C$ because of its simplicity and accuracy.

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