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EVALUATION OF HIGH SUGAR RYEGRASS VARIETIES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Animal and Veterinary Sciences

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by
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ABSTRACT

Annual ryegrass (*Lolium multiflorum*) is an important forage in Southeastern US. However, as in other winter annuals, concern exists about the balance between water soluble carbohydrates (WSC) and protein, which can lead to ruminal nutrient asynchrony. Lately, efforts have been done to increase the WSC content of ryegrass, and cultivars known as "high sugar varieties" have been released to the market. Several productive and environmental advantages have been suggested for these cultivars. The objective of our research was to compare conventional *versus* high sugar varieties under several conditions and from different approaches, to evaluate the potential of the high sugar varieties through a series of experiments. Our general hypothesis was that high sugar varieties would produce higher quality forage which in turn would lead to performance improvements and higher microbial protein synthesis. The first experiment evaluated four ryegrass varieties grown under greenhouse conditions varying in ploidy (diploid and tetraploid) and cycle length (annual or intermediate) to assess their chemical composition and digestibility. Later, two of those varieties which showed similar botanical characteristics and yields (Lonestar and Enhancer) were used in a two year experiment, to evaluate cattle performance, *in vivo* digestibility, and dry matter and forage intake, evaluating the interaction with corn supplementation as well. The next experiment involved the digestibility assessment of one of the varieties (Enhancer), grown in farm conditions, with four different *in vitro* methods: Daisy incubator system, batch culture, ANKOM gas production system and continuous culture fermenters, aiming to compare the results obtained by each of the *in vitro* method. In the last experiment, continuous culture

fermenters were used to evaluate the effect of WSC and soluble protein levels on fermentation parameters, microbial protein synthesis and nutrient digestibility. Additionally, this dissertation includes a literature review on the potential of residual feed intake for cattle production efficiency improvement.

DEDICATION

To my wife, Gabriela Volpi Lagreca, without whom I would have never achieved it

Thanks for the love, patience and support

To my daughter, Camila, who gave me hope and strength to keep working even in the hardest moments

To the new baby, Lautaro, for being the light at the end of the tunnel

ACKNOWLEDGEMENTS

Thanks to INTA, noble institution which gave me this opportunity

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CHAPTER ONE

LITERATURE REVIEW

ANNUAL RYEGRASS IMPORTANCE IN SOUTHEAST US BEEF PRODUCTION

Globally, grazinglands account for approximately four billion hectares. More than 300 million hectares are in the United States alone (Fisher, 2015). Eighty percent of the milk and 70% of the beef produced globally are from temperate grasslands (Wilkins and Humphreys, 2003). Even in the United States, where the feedlot industry is highly developed, forage represents on average 83% of the total nutrients consumed by beef cattle (Wilkins and Humphreys, 2003).

Annual ryegrass (*Lolium multiflorum* Lam.) is an annual cool-season bunchgrass originally from southern Europe which is currently cultivated throughout temperate zones on all continents (Jung et al, 1996). Among its characteristics, rapid germination and highly competitive seedling establishment make it a reliable option for producers. This is combined with high forage digestibility and palatability, which yields excellent animal performance in grazing stockers (Hannaway et al., 1999). In fact, some authors consider ryegrass as the most digestible of all the forage grass species available (Morrison, 1980, Frame, 1991).

The ideal conditions for annual ryegrass are cool and humid climates, with best growth rates occurring within the range of 20 to 25° C. It has a wide range of soil adaptability, both in terms of fertility, drainage and pH (Hannaway et al., 1999). In the United States, adequate growth conditions occur from the Atlantic coast to eastern parts of Texas and Oklahoma, as well as in some humid areas of Northwestern US. In the US, it covers an estimated surface of 1.2 million hectares, of which 90% is located in the Southeast (Hannaway et al., 1999). In fact in this area, where summer conditions impair perennial ryegrass (*Lolium perenne* Lam.) productivity and persistence, annual ryegrass is a preferable option for producers who use it as a winter annual and plant it in fall for grazing in winter and spring (Jung et al., 1996, Ball et al., 2007).

HIGH SUGAR RYEGRASS VARIETIES

In the last few years, there has been interest in the development of ryegrass cultivars with increased water soluble carbohydrate (WSC) content, also known as "high sugar grasses" or "high energy grasses" (Smith et al., 2007). These cultivars, when compared to conventional ones, show a higher concentration of fructans in leaf blade (Parsons et al., 2004; Edwards et al., 2007). Fructans are the main form of energy reserve in cool-season grasses. They are polymers or oligomers of fructose, branched or linear, linked to a sucrose core through a glycosidic bond (Rasmussen et al., 2014). In perennial ryegrass, three types of fructans have been detected: inulin series, inulin neoseries and levan neoseries, with the

majority (up to 76%) belonging to this last type. They consist mainly of fructose units linked by β-(2,6) glycosidic bonds (Rasmussen et al., 2014).

Many advantages have been suggested for high sugar grasses, both from a productive and an environmental standpoint. The most obvious one is the fact that an increased WSC content implies a higher digestibility and higher metabolisable energy (ME) content (Smith et al., 2007, Waghorn, 2007). Digestibility of WSC is higher than fiber digestibility, given that even in the youngest plants there is lignin cross-link formation which decrease availability of fiber for rumen microorganisms (Jung and Allen, 1995), whereas WSC are almost completely digestible.

High sugar grasses would also show an improved ruminal balance and synchrony of nitrogen and carbon supply (Edwards et al., 2007). Most of herbage protein is highly soluble, and thus rapidly degraded to ammonia in the rumen. Rumen microorganisms have the ability to use ammonia to synthesize microbial protein which is then available for absorption by the ruminant when the microorganisms reach the intestine (Cosgrove et al., 2007). However, if energy availability is not synchronized with ammonia availability, microorganisms are not able to capture the excess of ammonia and this is absorbed through ruminal wall, transported to the liver and converted into urea. This process implies a reduced efficiency in protein use, because most of this urea must be later excreted in urine, although some is recirculated through saliva and through rumen wall into the rumen again. Synchronous availability of protein and energy should allow a more efficient microbial protein synthesis, improving animal performance (Hall and Huntington, 2008). However, according to some evidence, readily available carbohydrates (i.e., WSC, starch) would be

a primary determinant of microbial protein synthesis irrespective of synchrony with ruminal protein availability (Hall and Huntington, 2008). Kim et al. (1999 a, b) found that microbial protein synthesis could be increased by carbohydrate infusion into the rumen either with or without synchrony with protein intake, because nitrogen availability would be provided by endogenous recirculation systems (i.e., urea in saliva), whereas availability of readily available carbohydrates would be the limiting factor for microbial synthesis. High sugar grasses would have the ability to reduce nitrogen losses through urine (Miller et al., 2001, Moorby et al., 2006), which would decrease soil and groundwater contamination as well as gaseous nitrous oxide emissions, which is known to be a greenhouse gas associated with global warming (Edwards et al., 2007).

When compared to conventional cultivars, some high sugar varieties have shown increased DM intake (Lee et al., 2002, Moorby et al., 2006). These would be explicable by a higher DM content in these cultivars (Miller et al., 2001, Moorby et al., 2006, Cosgrove et al., 2007) as well as by higher ruminal rates of degradation in rumen (Miller et al., 2001). Winter annuals in general, and ryegrass in particular, can have high moisture contents at vegetative stage, which in turn can affect DM intake by ruminants (John and Ulyatt, 1987). Numerous reports show that high sugar varieties have a higher DM content (Miller et al., 2001, Hopkins et al., 2002, Lee et al., 2002; Moorby et al., 2006, Cosgrove et al., 2007), which could partially explain the higher DM intakes observed in some trials (Moorby et al., 2006). On the other hand, an increased rate of degradation could reduce feed retention time in the rumen, reducing physical limitations to intake. Additionally, grasses richer in

sugars have shown to be more palatable, and intake of grazing ruminants appears to be sensitive to small changes in WSC content (Jones and Roberts, 1991, Mayland et al., 2005).

Differences in WSC content between control and high sugar cultivars range from 82 g/kg DM (Moorby et al., 2006) to small and non-significant differences (9 g.kg DM^{-1}) in one experiment carried out in New Zealand (Cosgrove et al., 2007). Actually, when evaluating exactly the same varieties at United Kingdom and at New Zealand, results differed widely between locations, mostly due to weather and fertility conditions (Edwards et al., 2007).

Cool-season grasses show both seasonal (Pollock and Jones, 1979) and diurnal (Mayland et al., 2005) patterns in WSC accumulation. Some research (Pollock and Jones, 1979) showed that WSC accumulation was higher during autumn and winter. Contrastingly, Cosgrove et al. (2009) reported higher concentrations of WSC in spring.

Sugar concentration also varies throughout the day, causing a circadian rhythm in forage quality (Mayland et al., 2005, Gregorini et al., 2006). During the light day period, photosynthesis causes a net increase in leaves' WSC concentration, while during the night photosynthesis stops and dark respiration consumes stored sugars and decreases total cell WSC content. This general pattern results in an increase in WSC content from sunrise to sundown and a subsequent decline during the dark night hours (Mayland et al., 2005). The increase in WSC is always accompanied by a decrease in the proportions of neutral detergent fiber (NDF) and acid detergent fiber (ADF) and, therefore, a more desirable forage composition from a nutritional standpoint. Indeed, Cosgrove et al. (2009) found that

per each unit increase in WSC (g/100 g), there was a decrease in NDF content which ranged between 0.30 and 0.17 g/100 g, depending on the season.

According to some evidence, temperature affects the expression of the high sugar trait. Experiments carried out in New Zealand (Parsons et al., 2004) showed that differences between high sugar and control cultivars were only significant when night temperatures were low (10° C) or when there was a previous phase of cold and short days. The authors suggested that lower night temperatures were necessary for the high sugar trait to express, because it would reduce the ratio of dark respiration to photosynthesis in plant tissues, allowing the accumulation of sugars (Parsons et al., 2004). The authors also concluded that this explained the inconsistent expression of high sugar trait in New Zealand compared to UK. Another experiment carried out in South Africa (Hopkins et al., 2002) compared two night/day temperature regimes (30°C/20°C vs 20°C/7°C) and found that in both regimes the high sugar varieties showed higher WSC content, but total content and difference between varieties were higher in the 20°C/7°C regime. Rasmussen et al. (2014) compared three varieties of high sugar perennial ryegrass versus a control variety at three temperature regimes (20◦C/20◦C, 20◦C/10◦C, or 10◦C/10◦C, light/dark temperature). They found that WSC content and fructosyltransferase expression was higher at low temperature. From all these results, it appears that genotype x environment interaction exists and that high sugar trait does not express equally in all environmental situations (Halling et al., 2004, Edwards et al., 2007).

From the above discussed, it would be interesting to evaluate the potential of high sugar annual ryegrass varieties and to assess their botanical behavior under the conditions of the southeastern US.

USE OF HIGH SUGAR RYEGRASS IN RUMINANT OPERATIONS

High sugar ryegrass varieties have been tested in dairy production. Early reports in the United Kingdom (Miller et al., 2001) showed that high sugar grasses improved milk yield in late lactation dairy cows without affecting milk solids composition. Interestingly, in this study neither total DM intake nor grass DM intake differed between treatments, but the differences in digestibility in favor of the high sugar variety led to a higher energy intake (Miller et al., 2001). These results coincided with lower urinary excretion of nitrogen and higher proportions of nitrogen secreted in milk. Other experience carried out later in the United Kingdom (Moorby et al., 2006) did not show any increase in milk yield, even though differences in WSC content between varieties were high. However, the authors reported (Moorby et al., 2006) higher milk protein yield in cows fed the high sugar grasses.

In general, results in terms of milk yield and milk composition have been variable and evidence is far from conclusive. While some reports show an interesting potential for increased dairy productivity using high sugar grasses (Miller et al., 2001, Tas et al., 2006), others report no differences (Moorby et al., 2006, Cosgrove et al., 2007). Tavendale et al. (2006) reported lower concentrations of undesirable aromatic compounds (skatole and indole) in rumen content and milk of cows fed high sugar grass. These compounds are

formed into the rumen from certain amino acids like the tryptophan and are related to low WSC:CP ratios, and their presence is associated with higher protein degradation in rumen.

There has been less research on the potential of high sugar grasses in meat production. Most of the experiments reporting results in meat production were conducted with sheep. Grazing lamb performance was evaluated in studies conducted in the United Kingdom. Lee et al. (2001) evaluated the performance of suckling lambs stocked either on a conventional or a high sugar *Lolium perenne* sward. They reported a significantly higher lamb production (kg/acre) from the high sugar variety due to increased liveweight gain and higher carrying capacity. The authors also found a strong correlation ($r=0.67$, $p<0.05$) between WSC content and liveweight gain. Marley et al. (2007) evaluated high sugar versus control varieties grazed either rotationally or continuously. High sugar grasses showed significantly higher WSC content and tended to produce higher liveweight gain ($P = 0.07$). The authors also found a higher serum total protein and a lower serum urea concentration, which led them to conclude that high sugar grasses increased nitrogen efficiency. More recently, interesting results were found by Evans et al. (2011), who evaluated performance of lambs grazing ryegrasses nominally differing in WSC content during pre-weaning and during post-weaning. The authors did not find any difference in WSC between varieties, although there was a lower NDF content and a higher DM digestibility in the high sugar variety. Lamb performance did not differ during the pre-weaning period, but was significantly improved in high sugar treatment during the post-weaning period.

Since most of the information available in the literature belongs to dairy systems or sheep production, it would be interesting to analyze the potential of high sugar ryegrass varieties in beef cattle production under the southeastern US conditions.

ENERGY SUPPLEMENTATION IN FORAGE BASED STOCKER SYSTEMS

The term supplementation defines the practice in which grazing cattle receive an additional input of stored feeds. According to Allden (1981), supplements can be classified into three basic groups: i) energy rich (i.e., grains), ii) protein rich (or non-protein nitrogen), and iii) inorganic nutrients (i.e., minerals). Reasons for supplementation will depend on production objectives, whereas animal response will vary with level and type of supplementation, type of animal, and quality and availability of basal forage. In particular, grain supplementation aims to increase the energy intake of grazing animal, either in situations of low forage availability or when forage nutrient content and balance is lower than the required for an expected performance (Horn and McCollum, 1987). In other cases, supplements can help to increase or maintain stocking rate during transitory forage shortages, adding to the stability and predictability of the enterprise, thus reducing production risks (Allden, 1981, Horn et al., 2005).

Grain supplementation generally decreases forage intake, which is known as substitution. The magnitude of substitution can be quantified by the substitution ratio (or substitution rate, Bargo et al., 2003), which is defined as the unit change in forage intake per unit increase in concentrate intake (Horn and McCollum, 1987, Bargo et al., 2003).

Substitution ratio is calculated as: (forage DM intake in unsupplemented treatment – forage DM intake in supplemented treatment) / supplement intake (Bargo et al., 2003). For example, if substitution ratio is 0.70, forage intake was reduced 0.70 kilograms per each kilogram of supplement consumed. In general, substitution ratio is usually lower than 1; therefore, total digestible DM intake typically increases (Waldo, 1986). Additionally, according to Jarrige et al. (1986), forages with greater potential intake (i.e., higher nutritional quality) will have greater substitution ratios. This is also reported by Horn and McCollum (1987), who reported that at forage digestibilities of 60, 65 and 75 g/kg^{-1} DM, substitution ratios were 0.1, 0.3 and 0.5, respectively.

Pordomingo et al. (1991) supplemented steers with different levels of shelled-corn (0, 0.2, 0.4 and 0.6% LW) and found lineal depression of forage intake, without changes in the total DM intake. Similarly, Pavan and Duckett (2007) reported that supplementation with cracked corn (0.52% LW) reduced forage intake without affecting total DM intake in steers grazing tall fescue.

Substitution rate is also dependent on forage availability (Allden, 1981), and several studies show that at higher pasture availability, the substitution rate increases (Bargo et al., 2002). French et al. (2001) found that at low forage availability (6 kg DM. $an^{-1}.d^{-1}$), supplementation did not affect forage intake, whereas, at higher levels of forage availability (12 and 18 kg DM. $an^{-1}.d^{-1}$), supplementing with concentrates led to a reduction in forage intake (0.43 and 0.81 kg forage DM per kg concentrate DM, respectively). Bargo et al., (2002) supplemented dairy cows grazing at two different pasture allowances (25 and 40 kg forage DM. cow^{-1}) and found a higher substitution rate in the high pasture allowance

treatment (0.26 vs 0.55, respectively). In general, as the substitution rate increases the individual response to supplementation decreases, both in dairy cows (Bargo et al., 2003) and in beef cattle (Beretta et al., 2006).

One of the effects often associated with grain supplementation is ruminal pH decrease. Ruminal pH affects the activity of cellulolytic microorganisms (Mould and Ørskov, 1983, Mouriño et al., 2001, Calsamiglia et al., 2008). Therefore, depending on the nature and level of supplementation, the type of forage, grain supplementation might impair forage digestibility. Ørskov and Fraser (1975) fed sheep a diet comprised of grass and barley, varying both the amount of grain (25 and 50 g. kg^{-1} MW) and its starch degradability through processing. The experiment showed that the higher dose of processed barley reduced both fiber digestibility and forage intake. They concluded this was due to the reduction in ruminal pH produced by the increased intake of highly degradable starch. Fiber degradation results declined at pH below 6.2, and were completely inhibited at pH below 6.0 (Mould and Ørskov, 1983, Disjktra et al., 2012). However, this effect does not appear to be related to a direct effect of the pH on the cellulase activity (Russell and Wilson, 1988). It rather seems that pH affects the ability of cellulolytic bacteria to grow and attach to substrate (Russell and Wilson, 1996, Sung et al., 2007)

Grain supplementation of grazing cattle has a variable impact on rumen pH (Berzaghi et al., 1996, Elizalde et al., 1998, Bargo et al., 2002). However, within the range of usual levels of supplementation, strong effects on pH would not be expected, at least not to the point of affecting fiber digestibility. Pordomingo et al. (1991) supplemented steers with different levels of shelled-corn (0, 0.2, 0.4 and 0.6% LW) and found no significant

effect on rumen pH. In this study even the higher levels of supplementation ruminal pH ranges between 6.0 and 6.4, with an average of 6.3. Elizalde et al., (1998) supplemented steers grazing tall fescue with cracked corn at 0.75% LW and reported no effect on ruminal pH. Similarly, Fieser and Vanzant (2004) supplemented cattle consuming fescue hay with coarse cracked corn at 0.67% LW and reported no effect on rumen pH, which on average was always above 6.0.

One of the most widely cited effects of grain supplementation in grazing cattle is the improvement of individual average daily gain (ADG) (Pavan and Duckett, 2007, Latimori et al., 2008, Del Campo et al., 2008, Wright et al., 2015). Del Campo et al. (2008) found a linear increase in ADG when supplementing steers with corn grain at 0.6% and 1.2% LW. Similarly, Latimori et al. (2008) supplemented steers with 1.0 % LW cracked corn and reported an increase in ADG. Pavan and Duckett (2007) reported higher ADG when steers rotationally grazing non-toxic tall fescue were supplemented with cracked corn (0.52 % LW basis). Wright et al. (2015) reported increased ADG in steers grazing either legumes or grasses, when supplemented with cracked corn at 0.75% LW.

However, effects on individual performance might depend on several factors, including level of supplementation. For example, Latimori et al. (2008) found no significant differences in ADG of steers supplemented with corn at 0.7% LW when compared with unsupplemented control, whereas those supplemented at 1.0% LW had higher ADG. Forage availability can also impact response to supplementation. For example, Beretta et al. (2006) reported a significant interaction between forage allowance and supplementation (1.0 % LW). At daily allowances of 3 and 6 kg forage DM.100 kg^{-1},

there was a positive effect of corn supplementation on ADG, whereas at forage allowances of 9 kg DM.100 kg^{-1}, there was no performance response to supplementation. As a consequence, supplement conversion was improved lower forage availabilities. However, French et al. (2001), working with three levels of forage availability (6, 12 and 18 kg DM. $an^{-1}.d^{-1}$), observed a positive effect of the concentrate supplementation on ADG, regardless of forage allowance. Probably, the difference between these two experiments is that French et al. (2001) used continental cattle with high gain potential, whereas Beretta et al. (2006) used moderate frame Hereford cattle. Other researchers have also reported that at high availabilities of high quality forage, individual improvements in performance are not always realized (Dodsworth and Ball, 1962, Steen and Kilpatrick, 1998). Supplement conversion ratio (expressed as kg of supplement intake per kg of extra gain) tends to increase as forage availability increases (Beretta et al., 2006), indicating that the marginal gain due to supplementation is lower as the forage availability increase. In fact, among the pasture factors that affect total forage DM intake, pasture mass (kg $DM.ha^{-1}$) and pasture allowance (kg DM.kg LW^{-1}) are considered the most important (Bargo et al., 2003). Forage intake increases asymptotically as pasture allowance increases, usually reaching the higher attainable performances at higher forage allowances (Sollenberger and Vanzant, 2011). At higher forage allowances, cattle can select higher quality forage (Ball et al., 2007), which might lead to the satisfaction of nutritional demands irrespective of supplementation.

Since grain supplementation effect varies widely depending on forage availability and composition, and WSC level in the forage might interact with starch supplementation,

we consider interesting to evaluate grain supplementation on cattle grazing high sugar ryegrass varieties.

THE USE OF INDIGESTIBLE MARKERS FOR FORAGE INTAKE ESTIMATION

Since DM intake is the main determinant of performance by grazing ruminants (Lippke, 2002), its estimation is clearly important. However, DM intake in grazing animals is more difficult to predict than in confinement systems (Bargo et al., 2003). The simplest method involves on assessment of the pre and post grazing forage mass, which by subtraction would help infer forage DM intake. Unfortunately, this method can only be used for collective rather than individual intakes (Bargo et al., 2003). Additionally, it makes forage lost by trampling, urine and feces contamination during the grazing period difficult to assess (Ball et al., 2007). Finally, it is only applicable to rotational grazing, not to continuous grazing situations.

Estimations through the use of indigestible markers allow for individual assessments of intake, based on the estimation of fecal output (FO) and diet digestibility (Lippke, 2002), as follows:

$$\text{DM intake} = \text{FO} \times \text{indigestibility}^{-1}$$

$$\text{Where indigestibility} = 1 - \text{DM digestibility}$$

An indigestible marker is a substance which is completely indigestible, therefore recovered in feces (Schneider and Flatt, 1975). Internal markers are indigestible fractions originally present in the feed, whereas external markers are substances added to the diet and not present in the original feed (Van Soest, 1994). Properties of a good marker are: 1) distinctly mark the feces resulting from the feed with which the marker was fed, 2) to be inert, with no toxic, physiological or laxative effect on the normal digestion process, 3) to be neither absorbed nor metabolized, 4) not to react with the nutrient or nutrients under investigation, and 5) allow for accurate and sensitive quantification in feces after recovery (Schneider and Flatt, 1975, Van Soest, 1994, Lippke, 2002).

External markers

Examples of external markers are chromic oxide (Huston et al., 1999), rare earths like Ytterbium, lanthanum, and terbium (Owens and Hanson, 1992), long chain alkanes (Mayes et al., 1986) and titanium dioxide (TiO_2,Titgemeyer et al., 2001, Myers et al., 2004). External markers can be dosed to the animal (i) in a single large pool at the beginning of the trial, (ii) in uniform daily pulses, (iii) using controlled release devices (Lippke, 2002). The objective is to obtain a constant release and a uniform concentration of the markers in feces, which allows for the estimation of the FO, as follows:

$$FO\ (kg\ DM.d^{-1}) = Daily\ dose\ (g) \times Marker\ concentration\ in\ feces\ (g.\ kg\ DM\ feces^{-1})^{-1}$$

Even though continual release is expected, both dosing and fecal sampling frequency are important: As the frequency of dosing and sampling increase, the diurnal variation in release decreases; however, multiple daily intervention could be impractical and disturbing for animals, affecting their normal grazing behavior (Lippke, 2002).

Chromic oxide has been the most widely used traditionally, although TiO_2 provides a similar fecal recovery, yielding digestibility estimations at least as accurate as chromic acid while showing some advantages from an environmental and health standpoint (Titgemeyer et al., 2001, Myers et al., 2006). Glindemann et al. (2009) performed several experiments to evaluate the use of TiO_2 to estimate DM intake and found that recovery was close to 1 (within the range of 0.96 to 1.09). The authors also concluded that the equilibrium in the marker release was reached after five days of administration and that to have an acceptable accuracy both dosing and sampling had occur at least twice daily. Titgemeyer et al. (2001) evaluated TiO_2 as indigestible marker. In the first study, digestibility results obtained from a total fecal collection experiment were similar to those obtained dosing 10 g of TiO_2 per day. In experiment 2, total fecal collection digestibility results were closer to those obtained using TiO_2 than those obtained using chromic oxide. Therefore, TiO_2 appears to be an acceptable external marker with certain advantages over chromic oxide. Additionally, a simple and inexpensive colorimetric technique has been developed to assess TiO_2 concentration in feces. Briefly, the technique consists on a sulfuric acid digestion followed by the addition of 30% hydrogen peroxide, which gives a colored reaction that shows absorbance at 410 nm (see further details on Myers et al., 2004).

Internal markers

As previously mentioned, internal markers are an indigestible constitutive part of the feed (Owens and Hanson, 1992). Examples of internal markers are lignin (Fahey and Jung, 1983), indigestible NDF and ADF (Lee and Hristov, 2013), acid insoluble ash (Lee and Hristov, 2013) and long chain alkanes (Mayes et al., 1986). They are used to estimate digestibility by assessing their feed:feces ratio (Schneider and Flatt, 1975), as follows:

$$\text{Digestibility (\%)} = 100 - (100 \times [\text{Marker feed}]/[\text{Marker feces}])$$

Some authors have suggested that *in vitro* digestibilities together with FO could be used to estimate DM intake in grazing situations (Cordova et al., 1978). However, *in vitro* digestibility can hardly mimic the multiple aspects which could affect *in vivo* digestibility, including rate of passage, level of intake and other associative effects (Cochran et al., 1986).

Long time *in vitro* or *in situ* incubations yield an indigestible residue which can be later treated with neutral detergent or acid detergent to yield indigestible NDF (iNDF) or indigestible ADF (iADF), respectively (Waller et al., 1980, Lippke 2002). Cochran et al. (1986) compared *in vivo* digestibilities assessed by total feces collection with those obtained with several internal markers, including iADF and iNDF, and found that the

recovery of these markers was superior than other internal markers and that, for some feeds, the correlation between *in vivo* estimations by total collection and by the internal markers (iNDF and iADF) was very good. Lee and Hristov (2013) compared iNDF with total collection and acid insoluble ash, and concluded that iNDF was a more reliable marker than acid insoluble ash. On the other hand, the results obtained by total collection, even though not identical, were similar to the results obtained with iNDF. With respect to lignin as internal marker, data indicate that it would be poorly reliable, suffering partial digestion and structural modification in the gastrointestinal tract, thus yielding a poor fecal recovery (Fahey and Jung, 1983). Even though none of the internal markers possesses all the characteristics of an ideal marker, iNDF seems to be an accurate and reliable marker, which could help to assess DM digestibility and forage DM intake in grazing experiments.

CONCLUSION

Given the importance of annual ryegrass in the southeast US, the evaluation of new varieties with improved nutritional characteristics (i.e., high sugar varieties) is an important area for research. Since WSC are highly soluble and fermentable, their interaction with different sources of energy (i.e., starch) or protein (i.e., urea) would give further insights on the potential of high sugar ryegrass varieties for increasing productivity and improving grazing systems sustainability and profitability.

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CHAPTER TWO

CHEMICAL COMPOSITION AND IN VITRO DIGESTIBILITY OF HIGH SUGAR AND CONVENTIONAL RYEGRASS VARIETIES GROWN IN GREENHOUSE CONDITIONS

ABSTRACT

Plant breeders have recently focused on increasing the sugar content of grasses as a means to improve their nutritional value. The objective of this study was to compare the chemical composition of four annual ryegrass varieties *(Lolium multiflorum* Lam.*)*: two intermediate tetraploids [Bandito2, (conventional) and Abereve, (high sugar)] and two annual diploids [Lonestar, (conventional) and Enhancer, (high sugar)] grown in greenhouses. Seeds were planted into plastic (3.84 L) pots (16 pots per variety), hand watered daily and fertilized weekly with water soluble 20-10-20 (N-P-K). A total of three harvests (5 cm cutting height) were clipped at six-week intervals. All harvests were taken at 2:30 PM on days with full sunlight. Material was weighed, flash frozen, lyophilized and ground (1 mm). Chemical analyses included assessment of DM, OM, NDF, ADF, ADL, WSC and CP. Hemicellulose, cellulose content and WSC:CP ratio were calculated. Digestibility at 24 and 48 h was assessed using a Daisy^{II} incubator. *In vitro* DM, OM and NDF disappearance as well as *in vitro* true DM disappearance were calculated. Results were compared by preplanned orthogonal contrasts as follows: C1, intermediate tetraploids vs annual diploids, C2, conventional vs high sugar varieties. Intermediate tetraploid

varieties had lower DM content, lower OM content, lower NDF and hemicellulose content. They also tended to have higher CP content, but no differences were observed in WSC content or WSC:CP. Conventional and high sugar varieties did not differ except for DM content. Intermediate tetraploid had higher *in vitro* DM and OM disappearance at 24 and 48 h, and higher *in vitro* true DM disappearance and NDF disappearance at 24h. Conventional varieties had higher digestibility at 24 h but not at 48 h. No differences in WSC were detected between intermediate tetraploids and annual diploids, or between conventional and high sugar varieties. Differences in forage quality were more important between intermediate tetraploids and annual diploids, but no differences were found between conventional and high sugar varieties. Temperatures present in the greenhouse environment may not have allowed the potential of high sugar varieties to accumulate increased levels of water soluble carbohydrates.

INTRODUCTION

Annual ryegrass (*Lolium multiflorum* Lam.) is an annual cool season grass cultivated throughout all temperate zones around the world (Jung et al., 1996, Wilkins and Humprey, 2003). Due to its high digestibility and palatability, which yields excellent animal performance, it is used as forage source in cattle with high nutrient requirements. However, forage grasses do not always provide an appropriate nutrient balance. Low water soluble carbohydrate (WSC) content or low WSC to crude protein ratios (WSC:CP) can lead to a nutrient imbalance which impairs the ability of ruminal microorganisms for synthetizing microbial protein, leading to the absorption of ammonia through the rumen

wall. Its conversion to urea in the liver energy consuming and nitrogen is partially lost by urine (Nocek and Russell, 1988, Kingston-Smith and Theodorou, 2000). Therefore, an improved nutrient balance in grasses (i.e., a higher WSC:CP ratio) could potentially lead to higher nitrogen use efficiency, thereby increasing the quantity of microbial protein flowing to the intestine of the host animal. Additionally, this offers environmental benefits due to a reduction in nitrogen leaching into soil and groundwater as well as a reduction in emissions of nitrous oxide, which is known to be a greenhouse gas associated with global warming (Edwards et al., 2007).

With the aim of increasing the sustainability and profitability of grazing systems, plant breeders have lately developed forage varieties high in WSC, known as high sugar grasses or high energy grasses (Smith et al., 2007). Tetraploid and diploid varieties which express higher concentration of fructans in leaves may offer productive advantages for producers. Tetraploids cultivars are associated with higher levels of WSC as well as a higher cell content to cell wall ratio (Hageman et al., 1993).

Miller et al. (2001) reported milk yield improvement in late lactation dairy cows without affecting milk solids composition in cows grazing high sugar perennial ryegrass *(Lolium perenne)*. These results coincided with lower amounts of nitrogen excreted in urine and higher proportions of nitrogen secreted in milk. Moorby et al. (2006) found higher DM intake, higher DM digestibility, improved microbial protein synthesis and a higher protein yield in dairy cows fed high sugar ryegrass. Lee et al. (2001) evaluated the performance of suckling lambs stocked either on a conventional or a high sugar *Lolium perenne* sward and found a significant higher lamb production in the high sugar sward due to an increased

liveweight gain and higher carrying capacity. The authors reported a strong correlation ($r=0.67$, $p<0.05$) between WSC content and liveweight gain.

To our knowledge, most of the published research was carried out evaluating perennial ryegrass. However, perennial ryegrass is not viable in many locations where high summer temperatures reduce photosynthesis efficiency and increase respiration rates, affecting the summer survival of the plants. In those places (i.e., southeastern United States), annual ryegrass is a preferable option for producers, who use it as a winter annual, planting it in fall for grazing in winter and spring (Jung et al., 1996). However, scientific information is scarce for high sugar annual ryegrass varieties, and the ability of this species to accumulate WSC has only been tested by a smaller number of researchers (Hopkins et al., 2002).

The objective of this study was to analyze chemical constituents that affect nutritive value and *in vitro* digestibility of four ryegrass varieties: two intermediate tetraploids [Bandito2, (conventional) and Abereve, (high sugar)] and two annual diploids [Lonestar, (conventional) and Enhancer, (high sugar)] grown in greenhouse conditions. Our hypothesis was that intermediate tetraploids and high sugar varieties would have higher WSC content, lower cell wall concentration and higher *in vitro* digestibility.

MATERIALS AND METHODS

The experiment was conducted at a 15 m x 13 m greenhouse at Clemson University, Clemson, South Carolina, USA, between January and June 2013. Seeds of annual ryegrass (*Lolium multiflorum* Lam.) were planted at 0.5 cm depth into plastic pots (18 cm height x

15 cm diameter x 15 cm deep) containing potting soil, on top of 0.80 m height metal tables. A total of four varieties were evaluated: two intermediate tetraploids [Bandito2, (conventional) and Abereve, (high sugar)] and two annual diploids [Lonestar, (conventional) and Enhancer, (high sugar)]. All the varieties were provided by Sucraseed (OR, USA). Sixteen pots per variety were planted. After planting, pots were watered to saturation and, after germination, plants were watered daily with tap water and fertilized weekly with 20-10-20 (N-P-K) Peters professional plant nutrient solution (Scotts Sierra Horticultural Products Company, Marysville, OH). No artificial light was used, so the daylight length increased from the beginning to the end of the experiment. The greenhouse was equipped with an airflow distribution system. Temperature varied from a minimum of 18º C during the night to a maximum of 29ºC during the day and relative humidity was maintained at 70%.

Plants were harvested at six-week intervals by clipping at 5 cm height. A total of three cuttings were harvested (March 7th, April 18th and May 30th). All harvests started at 2:30 PM on days with full sunlight, to ensure a higher accumulation of WSC (Mayland et al., 2005). Material was immediately weighed, placed in cloth bags, and flash frozen in liquid nitrogen. Plant material was stored at -20 C until lyophilized (Labconco 7806021 bulk tray dryer, USA), ground through a Wiley mill to pass a 1 mm screen, except for an aliquot which was used to estimate dry matter content (DM) by drying in the oven at 102º C until constant weight. The freeze dried material was then pooled by variety and sampling. Plant tissue analyses included ash content by placing on muffle furnace (600º C, 6 h), NDF and ADF content which were assessed in the ANKOM fiber analyzer according to Van

Soest et al., (1991), acid detergent lignin (ADL) by immersing samples into 72% H_2SO_4 (Van Soest et al., 1991), water soluble carbohydrate content (WSC) was assessed by colorimetric phenol-sulfuric acid assay according to Dubois et al. (1956), crude protein concentration by combustion method on a Leco FB528 analyzer (Leco Corp., St. Joesph, MI; AOAC, 1990). Hemicellulose was estimated as the difference between NDF and ADF, and cellulose as the difference between ADF and ADL (Van Soest et al., 1994)

For the estimation of the *in vitro* DM, OM and NDF disappearances, dry and ground forages (0.50±0.01 g) were weighed into acetone pre-rinsed incubation bags (F57 bags, Ankom, Fairport, NY, USA) in duplicate for each variety and sampling. Then they were incubated in a Daisy^{II} *in vitro* incubator (Ankom, Fairport, NY, USA). This system consists of an incubator maintained at 39.5 º C by means of a light bulb controlled by a thermostat, where glass jars rotate at 0.95 rpm by means of gear drives (Robinson et al., 1999). Rumen fluid was collected from a cannulated Holstein dairy cow in mid lactation fed a diet comprised of 34% corn silage, 6% grass hay and 60% grain mix diet. Liquid and fistfuls of fibrous material were collected from the dorsal and ventral sac of the rumen, kept in pre-warmed thermic bottles and taken to the lab, where it was blended in a in a preheated blender while purged with CO_2, for 30 seconds. A total of 400 ml of the filtered rumen fluid was poured into the incubation jar that contained 1600 ml of buffer (KH_2PO_4, 8.3 g/l, $MgSO_4*7H_2O$, 0.41 g/l, NaCl, 0.41 g/l, $CaCl_2*2H_2O$, 0.08g/l, urea 0.41 g/l, Na_2CO_3 , 2.5 g/l and Na_2S*9H_2O, 0.16 g/l) while purging with CO_2. *In vitro* true digestibility (IVTD) was obtained by calculating NDF content in the residue post incubation (Goering and Van Soest, 1970).

Statistical Analyses. Chemical composition variables were analyzed by Proc Glimmix of SAS (SAS Institute, Cary, NC) in a model that included variety as fixed factor and cutting date as a random factor. Two pre-planned orthogonal contrasts were used for comparisons: C1, to compare intermediate tetraploids (Bandito2 and Abereve) vs. and annual diploids (Lonestar and Enhancer), C2, to compare conventional (Lonestar and Bandito2) vs high sugar (Enhancer and Abereve). Differences between means with $P<0.05$ were considered to be statistically different, while differences with $P<0.10$ were considered as tendencies.

RESULTS AND DISCUSSION

Forage chemical composition is shown in Table 2.1. The DM content of the intermediate tetraploid varieties was lower than that of the annual diploid varieties. This differences, obtained under identical environmental conditions and at the same growing intervals, would indicate genetic differences between the types. Several authors have reported that tetraploids grasses have lower DM content than diploid grasses (Van Wijk, 1988, Baert, 1994, Wims et al., 2012). Additionally, maturation is faster in annual varieties, reaching a higher DM content compatible with a more advanced phenological stage. Intermediate varieties are crosses of annual x perennial varieties; therefore, they show characteristics intermediate between those two (Hannaway et al., 1999). The two high sugar varieties (Abereve and Enhancer) tended ($P = 0.06$, Table 2.1) to have higher DM content than conventional varieties (Bandito2 and Lonestar). Higher DM contents in high sugar ryegrass varieties have been reported by several authors (Miller et al., 2001, Moorby

et al., 2006, Cosgrove et al., 2007). Even though it does not affect the ruminal digestion process, DM content of grasses is a factor affecting animal performance, because higher DM content of forages could improve voluntary intake (John and Ulyatt, 1987).

Diploid annual had a higher OM content than diploid intermediates, while no differences were found in the contrast between high sugar and conventional varieties. With respect to the cell wall components analysis, intermediate tetraploid tended to have lower NDF content ($P = 0.07$), with the hemicellulose fraction being significantly lower (Table 2.1). Since no differences were observed in ADF content, the cellulose fraction resulted higher in the intermediate tetraploid. No differences were found in ADL (Table 2.1). The contrast between high sugar varieties and conventional varieties did not differ. Lower NDF (Wims et al., 2012) and lower hemicellulose content (Morrison, 1980) in tetraploid varieties have been previously reported. The duplication of chromosome number in tetraploid varieties is associated with increased cell size and higher cell content to cell wall ratio, which have a dilution effect on NDF concentration (Hageman et al., 1993). Fiber concentration and digestibility are usually correlated (Wilkins and Humphreys, 2003). Additionally, fiber concentration, due to its filling effect, could be more important that DMD in determining forage intake and animal performance (Wilkinson et al., 1982).

Crude protein content tended to be higher in the intermediate tetraploid varieties ($P = 0.09$, Table 2.1). This agrees with the reports of Cosgrove et al. (2009) and Wims et al. (2012) who reported that tetraploid perennial ryegrass varieties at vegetative stage had higher CP content than diploids. No differences in WSC were found between intermediate tetraploids and annual diploids (Table 2.1). Water soluble carbohydrates and CP and are

the main components of the cell content (Wilkins and Humphreys, 2003). As previously mentioned, tetraploid grasses have higher cell content. This is in turn associated with higher WSC and CP content, as well as proteins and lipids, and improvements in forage digestibility (Hageman et al., 1993, Nair, 2004).

Research has shown that the expression of the high sugar trait is affected by environmental conditions (Parsons et al., 2004, Cosgrove et al 2014). For example, Parsons et al. (2004) reported that high sugar varieties of perennial ryegrass were not different from conventional varieties when grown in pots outside and only showed slight and inconsistent differences when grown in paddocks in a field trial. Additionally, the authors ran experiments under controlled conditions in growth chambers and found that the expression of high sugar trait was more strongly expressed when either low night temperatures or long previous periods of low temperatures were present. The authors suggested that low temperatures would reduce the ratio of dark respiration to photosynthesis in plant tissues, allowing the accumulation of sugars (Parsons et al., 2004). Cosgrove et al (2007) reported slight differences (2 to 4 g/ kg DM, depending on the year) between high sugar grasses (diploid and tetraploids) and conventional varieties in spring, but no significant differences when the same varieties were compared in fall. Conversely, working at field paddocks in New Zealand, Lazzarini et al. (2010) found no differences between varieties in spring and slight differences (1.5 g/ 100 g DM) in fall. Rasmussen et al. (2014) detected effects of growth temperature not only on the ability of varieties to concentrate WSC, but also on the expression of specific fructosyltransferases, which showed a reduced expression at high temperatures. The abovementioned results show that genotype x environment interaction

exists in the expression of high sugar trait, which does not express equally in every environmental situation (Halling et al., 2004, Edwards et al., 2007, Rasmussen et al., 2014). In our experiment, temperature varied between 18º C and 29ºC, which could have impaired the expression of the high sugar trait in our varieties.

Given that DM yield is an important factor affecting productivity of forage based systems, results of DM yield in g DM. pot^{-1} are reported in Table 2.1; however, these results should be taken carefully, because they may not be directly extrapolated to field situations. Tetraploids showed lower DM yield than diploids, consistent with findings of Balochi and López (2009). This could be at least partially explained by the lower DM concentration in the intermediate tetraploid varieties (Table 2.1).

With respect to *in vitro* disappearance and digestibility data (Table 2.2), intermediate tetraploid varieties tended to have higher IVDM disappearance both at 24 and 48 h of incubation ($P = 0.10$ and $P = 0.08$, respectively) and significantly higher IVOM disappearance at both incubation times ($P < 0.05$). At 24 h, DM IVTD and IVNDF disappearance were also higher in intermediate tetraploids, but there were no differences at 48 h of incubation (Table 2.2). These results agree with those obtained by Skaland and Volden (1973) in Norway, Wims et al (2012) in Ireland, and Balochi and López (2009) in Chile, who reported that tetraploid varieties had higher digestibility.

Regarding the contrast between conventional and high sugar varieties (C2, Table 2.2), results indicate that conventional varieties had higher IVDM disappearance, IVOM disappearance, DM IVTD and IVNDF disappearance at 24 h of incubation. These differences disappeared for all variables at 48 h of incubation (Table 2.2). With no

differences in composition (Table 2.1) between conventional and high sugar varieties, results may be explained by a more quickly digestible fiber fraction in the conventional varieties, especially Bandito2.

Ryegrass is the most digestible of all the grass species that have been tested (Morrison, 1980, Frame, 1991). We report average IVDM disappearance values of 71.02 g/100 g DM and 82.15 g/100 g DM at 24 and 48 h of incubation, which are close the values reported by Hopkins et al. (2002). Acid detergent lignin values were very low (2.27 g/100 g DM, on average), which helps to explain the high digestibility (Jung and Allen, 1995, Moore and Jung, 2001).

CONCLUSION

Both in terms of chemical compositions and *in vitro* disappearance and digestibility, intermediate tetraploids showed parameters compatible with higher nutritive quality. Either no differences or minor significant differences were found when comparing conventional to high sugar varieties. No variety effect was detected in WSC content, possibly due to higher than optimal temperatures which might have impaired the expression of the high sugar trait. Breeding strategies for high WSC varieties should include the selection of genotypes with the ability to concentrate WSC in a wide range of environments, including warmer temperatures (Rasmussen et al., 2014).

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Table 2.1. Dry matter yield and chemical composition of ryegrass varieties (Bandito2, Abereve, Lonestar and Enhancer) grown in greenhouse conditions

	Intermediate Tetraploid		Annual diploid			*Contrasts*	
	Bandito2 (C)	Abereve (HS)	Lonestar (C)	Enhancer (HS)	SEM	*C1*	*C2*
Yield (g DM/ pot)	9.57	8.57	10.61	10.33	1.252	0.04	0.30
Composition *							
DM content ($g.kg^{-1}$ wet)	14.69	16.62	17.98	17.95	2.056	<0.01	0.06
OM content	90.21	90.31	90.82	90.78	0.915	0.02	0.89
NDF	45.33	44.66	46.04	45.90	1.396	0.07	0.39
Hemicellulose	16.98	17.01	18.75	18.23	1.221	<0.01	0.38
ADF	28.35	27.65	27.29	27.67	0.799	0.11	0.57
Cellulose	26.19	25.37	24.93	25.41	0.732	0.03	0.47
ADL	2.16	2.28	2.36	2.26	0.521	0.31	0.95
Crude protein	10.24	10.81	9.93	10.00	2.182	0.09	0.30
WSC	12.98	15.63	15.32	13.97	1.456	0.60	0.33
WSC:CP	1.37	1.63	1.75	1.55	0.388	0.16	0.73

* Presented as $g.kg^{-1}$ DM unless stated otherwise.

C: conventional, HS: high sugar.

DM: dry matter, OM: organic matter, NDF: neutral detergent fiber, ADF: acid detergent fiber, ADL: acid detergent lignin, WSC: water soluble carbohydrates, WSC:CP: water soluble carbohydrates to crude protein ratio. SEM: standard error mean. C1: orthogonal contrast intermediate tetraploid varieties vs annual diploid. C2: orthogonal contrast high sugar varieties (Abereve and Enhancer) vs conventional (Bandito2 and Lonestar).

Table 2.2. *In vitro* dry matter and organic matter disappearance, *in vitro* dry matter true digestibility and *in vitro* NDF disappearance at 24 and 48 hours of incubation of ryegrass varieties (Bandito2, Abereve, Lonestar and Enhancer) grown in greenhouse conditions.

	Intermediate Tetraploid		Annual diploid			*Contrast*	
	Bandito2 (C)	Abereve (HS)	Lonestar (C)	Enhancer (HS)	SEM	*C1*	*C2*
24 h incubation							
IVDM disappearance	73.12	70.35	71.32	69.30	2.375	0.10	0.009
IVOM disappearance	81.08	77.93	78.57	76.40	3.128	0.04	0.008
DM IVTD	81.58	77.40	77.83	77.32	2.279	0.02	0.005
IVNDF disappearance	59.55	49.67	52.15	51.02	3.979	0.06	0.002
48 h incubation							
IVDM disappearance	83.30	82.43	81.00	81.87	1.964	0.08	1.00
IVOM disappearance	92.37	91.38	89.27	90.22	2.895	0.03	0.98
DM IVTD	88.65	87.48	86.62	87.17	1.926	0.11	0.67
IVNDF disappearance	75.02	72.13	71.17	72.09	2.071	0.20	0.51

Presented as $g.kg^{-1}$ DM

C: conventional, HS: high sugar.

IVDM: *in vitro* dry matter disappearance after incubation in Daisy^{II}, IVOM: *in vitro* organic matter disappearance after incubation in Daisy^{II}, DM IVTD: dry matter *in vitro* true digestibility. IVNDF: *in vitro* neutral detergent fiber disappearance after incubation in Daisy^{II}. SEM: standard error mean. C1: orthogonal contrast intermediate tetraploid varieties vs annual diploid; C2: orthogonal contrast high sugar varieties (Abereve and Enhancer) vs conventional (Bandito2 and Lonestar).

CHAPTER THREE

PERFORMANCE AND DRY MATTER INTAKE OF ANGUS STEERS GRAZING TWO VARIETIES OF ANNUAL RYEGRASS, WITH OR WITHOUT CORN SUPPLEMENTATION

ABSTRACT

The objective of this study was to compare animal performance, forage intake, DM intake, and *in vivo* DM digestibility of annual ryegrass varieties (*Lolium multflorum* Lam.) selected to differ in water soluble carbohydrate (WSC) content [Lonestar, (conventional) vs Enhancer, (high WSC)], with and without cracked corn supplementation (0 or 0.67% BW). The experiment was conducted in two consecutive years (lasting 72 d in yr 1 and 112 d in yr 2), using 24 Angus steers each year (initial BW = 364 ± 15.9 kg yr 1, 316 ± 32.2 kg yr 2). The treatment design was a 2 × 2 factorial arrangement of variety and corn supplementation. Variety was applied in a completely randomized design with paddock per year as the experimental unit. Corn supplementation was applied in a split-plot design with steer as the experimental unit. Average daily gain (ADG) was calculated by difference between initial and final weight. Forage analysis included OM, WSC, crude protein, NDF and ADF content. During the second year, titanium dioxide (TiO_2) was used to estimate forage and DM intake. Indigestible NDF was used as internal marker to estimate DM digestibility. Forage chemical composition did not differ ($P > 0.05$) between varieties. No differences due to variety or supplementation were observed in final weight or ADG ($P >$

0.05). Additionally, in year 2, no differences due to variety or supplementation were found in forage intake, DM intake or *in vivo* DM digestibility ($P > 0.05$). Under the conditions of this study, no differences in forage composition, animal performance or forage digestibility were detected between the high sugar ryegrass variety and a conventional variety, or due to corn supplementation.

INTRODUCTION

The southeastern US is able to produce high quality forage year round, which gives ideal conditions for grass based cattle production (Kerth et al., 2007, Scaglia et al., 2014). In this area, tall fescue is the most important forage for livestock systems, but since its quality and productivity declines during the winter, it is necessary to use of alternative forages. Winter annuals (i.e., annual ryegrass, oat, cereal rye) are options for high quality forage production during winter. However, winter annuals do not always provide an appropriate nutrient balance, due to potential ruminal asynchrony in nitrogen and carbon chain supplies (Johnson, 1976, Hall and Huntington, 2008).

Several strategies have been proposed in order to overcome this constraint. Energy supplementation (i.e., grains) has been used not only as a way of providing a more balanced nutrient supply (Horn et al., 2005) but also to take advantage of substitution, aiming to increase or maintain stocking rates in periods of forage scarcity (Horn and McCollum, 1987, French et al., 2001). Pavan and Duckett (2007) reported that corn supplementation (0.50 % BW) reduced forage intake without affecting total DM intake, increasing average

daily gain (ADG) in rotationally grazed steers. Wright et al. (2015) supplemented steers grazing both grasses and legumes and found a higher ADG and hot carcass weight in the supplemented group.

On the other hand, the use of ryegrass varieties with increased water soluble carbohydrate (WSC) content has been suggested as a potential way of reducing urinary nitrogen losses and improving performance in dairy cows (Miller et al., 2001). Additionally, research suggests that higher WSC content could lead to improved performance in beef cattle (Gregorini et al., 2006). However, little is known about the productive results of stockers grazing high sugar cultivars in the Southeastern US. The objective of this experiment was to evaluate performance (ADG) and DM intake of Angus steers grazing varieties of annual ryegrass selected for contrasting WSC content, with or without cracked corn supplementation.

MATERIAL AND METHODS

An experiment was conducted in two consecutive years with the objective of evaluating the effects of annual ryegrass variety (*Lolium multflorum* Lam., variety: Lonestar [conventional] vs Enhancer [high sugar], Sucraseed, OR, US), with or without daily cracked corn supplementation (no supplement vs 0.67% $BW.d^{-1}$), on animal performance. Additionally, during the second year, DM intake, forage intake and DM *in vivo* digestibility was assessed using markers. Each year, 24 Angus steers (initial BW = 364 ± 15.9 kg year 1, 316 ± 32.2 kg year 2). The treatment design was a 2 × 2 factorial

arrangement of variety and corn supplementation. Variety was applied in a completely randomized design with paddock per year as the experimental unit. Corn supplementation was applied in a split-plot design with steer as the experimental unit. In each year, four paddocks (2 ha) were used, and steers were randomly assigned to paddock (6 steers per paddock). Within each paddock (2 replicates per variety per year), steers were randomly assigned to corn supplementation, with 3 steers not supplemented and 3 steers supplemented with cracked corn at 0.67% BW basis. Steers were supplemented individually using Calan gate feeders (American Calan Inc., Northwood, NH). Prior to the experiment, steers were trained in the use of Calan gate feeders in dirt-floor pens, feeding them ad libitum with a mix of bermudagrass *(Cynodon dactylon)* hay and soybean hulls. All steers used were originated at the Clemson University Simpson Research Farm. All experimental procedures were reviewed and approved by the Clemson University Institutional Animal Care and Use Committee.

To reduce variability due to digestive tract fill, steers were weighed unfasted on two consecutive days at the beginning and at the end of the experiment, and the average was used as initial and final BW, respectively. Average daily gain was estimated dividing the difference between the final and initial BW by the total days of experiment. Additional weights were collected at 28 d weight intervals to monitor weight performance, but they were not used for ADG estimation. Supplementation consisted of cracked corn fed at 0.67% BW basis, offered individually using Calan gates once daily at 0800 h. Corn amount was adjusted every 28 d according to individual steers weight. Steers were provided with shade and free choice mineral supplement (Ca 10.00%, P 10.00%, ClNa 11.50%, Mg

14.00%, Co 15 ppm, Cu 800 ppm, I 90 ppm, Mn 2000 ppm, Se 18 ppm, Zn 3000 ppm). No anabolic implants or ionophores were used in this experiment.

The experiment was conducted at Clemson University Simpson Farm (34º 37' 21" N, 82º 43' 33" W, Pendleton, South Carolina, US). In mid-October each year, 2 paddocks of each variety were established in 18 cm rows with either conventional annual ryegrass (variety Lonestar, Sucraseed, Tanger, Oregon, US) or high sugar annual ryegrass (variety Enhancer, Sucraseed, Tanger, Oregon, US). Paddocks were treated with glyphosate prior to seeding and fertilized at emergence with urea at a rate of 40 kg nitrogen. ha^{-1}.

During the first year of experiment, harsh winter climatic conditions delayed the start of grazing until the month of March, whereas during the second year grazing could be started in early February. During the grazing period, forage samples were collected every 14 d intervals cutting with clippers at 5 cm height. Samples were immediately placed on ice and transported to laboratory, where they were frozen at -20ºC, until drying at 60ºC for 48 h. Samples were ground in a Wiley mill to pass a 1 mm screen. Analysis included ash and OM content (600^0c for 6 h), NDF and ADF, which were assessed in the ANKOM fiber analyzer according to Van Soest et al., (1991), WSC content was assessed by colorimetric phenol-sulfuric acid assay (Dubois et al., 1956, Hall, 2013), and crude protein (CP) concentration by combustion method on a Leco FB528 analyzer (Leco Corp., St. Joesph, MI).

Forage availability was estimated at 14 d intervals using an electronic rising-plate meter (Farmworks F300, Aghub, NZ). Calibrations were developed at 6 weeks intervals.

The paddocks were grazed by continuous grazing throughout the whole growing season. Grazing started when forage accumulation exceeded 1000 kg DM.ha^{-1}. A put and take grazing system, using extra grazers when needed, was utilized to maintain forage availability between 100 and 180 kg DM.100 kg BW^{-1}, but extra grazers were not used in the performance calculations.

In the second year of the experiment, forage intake, total DM intake and DM digestibility was estimated using titanium dioxide (TiO_2) as external marker (Titgemeyer et al., 2001, Myers et al., 2004) to estimate fecal output (FO) and indigestible NDF to estimate diet digestibility (Lee and Hristov, 2013). Animals were restrained in a chute and dosed twice a day with 10 g TiO_2 total (5 g each time, at 8:00 h and 17:00 h) during ten days, using gelatin capsules as vehicle. Fecal samples were collected by rectal grab sampling twice a day at the same times during the last 3 days of dosing (days 8, 9 and 10). Fecal samples were dried at 60°C for 96 hours and ground in a Wiley mill to pass a 1 mm screen. The objective of dosing during ten days is to obtain a constant release and a uniform concentration of the markers in feces, which allows for the estimation of the FO, as follows:

$$FO\ (g\ DM.d^{-1}) = \text{Daily marker dose (g)} \times \text{Marker in feces (g. 100 g DM feces}^{-1})^{-1}$$

Fecal TiO_2 concentration was estimated according to the technique described by Myers et al. (2004). Briefly, samples were digested in concentrated H_2SO_4 for 2 h (Kjeldahl digestion), followed by addition of 15 ml of 30% H_2O_2, which gives a visible colorimetric

reaction within the green to yellow range. Absorbance was measured at 410 nm in a Biotek Synergy HT Multi-plate reader (Biotek, Winooski, VT, USA) .

Indigestible NDF (iNDF) content was used as internal marker to assess *in vivo* DM digestibility, as follows:

$$\text{DM Digestibility (\%)} = 100 - (100 \times [\text{iNDF feed}]/ [\text{iNDF feces}])$$

Feed and feces iNDF content was assessed by incubating dry and ground (1 mm) material in a DAISY^{II} (Ankom Technology, Fairport, NY) incubator during 120 h, and then measuring NDF content of the residue post-incubation in an ANKOM 2000 analyzer (Ankom Technology, Fairport, NY).

Finally, DM intake was estimated as follows:

$$\text{DM intake} = \text{FO} \times \text{DM indigestibility}^{-1}$$

Statistical analyses. Treatments were assigned according to a 2 x 2 factorial arrangement, with ryegrass variety and supplementation as factors. For ADG and final weight, variety was assigned in a completely randomized design with paddock as the experimental unit, whereas supplementation was applied in a split-plot design with steer as the experimental unit. A mixed model was developed with variety, corn supplementation, and the 2-way interaction as fixed effects and 2 different error terms. The error term for testing forage was the random effect of paddock within variety and the error term for testing

corn supplementation and corn supplementation x variety interaction was the random effect of animal within supplementation and variety combination. Initial BW was used as a covariate. Total DM intake, forage intake and DM digestibility were analyzed with a model that included variety, supplementation, the variety x supplementation as fixed factors, and paddock within variety as random factor. Grass chemical composition, availability and allowance were analyzed with a model that included variety as fixed factor, and year, sampling time and paddock within variety as random factors. All the analyses were performed using the GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC, US). Differences were considered significant when $P<0.05$, and differences with $P > 0.05$ to $P< 0.10$ were considered as tendencies.

RESULTS AND DISCUSSION

There was no effect of ryegrass variety or corn supplementation on ADG and final BW ($P > 0.05$, Table 3.1). Individual response to grain supplementation is dependent on several factors. Among them, forage availability (kg DM. ha^{-1}) and allowance (kg DM.100 kg BW^{-1}) play an important role. Steen and Kilpatrick (1998) found that supplementing cattle at contrasting forage availabilities improved individual performance only at low availabilities. Similarly, Dodsworth and Ball (1962) reported no effect on individual ADG when concentrate supplement was fed to steers grazing a sward with high availability and high quality. In our experiment, availability was 1608.2 and 1627.2 kg DM. ha^{-1} for Enhancer and Lonestar, respectively (Table 3.2). More importantly, forage allowance was

122.51 and 131.86 kg DM. 100 kg BW^{-1}, for Enhancer and Lonestar, respectively. Another factor to be considered in the individual effect of supplementation is grazing method. In rotational grazing situations, cattle are forced to graze less digestible portions of the grass (i.e., pseudostems). Continuous grazing with high forage availability allows for selection of more palatable and digestible parts of the plant (Ball et al., 2007). Ryegrass is one of the most highly digestible grasses (Morrison, 1980, Frame, 1991); therefore, it should be expected that under continuous grazing conditions and without limitations to forage intake, both supplemented and unsupplemented steers have the ability to select a high quality overall diet, reducing the potential benefit of energy supplementation (Ball et al., 2007, Sollenberger and Vanzant, 2011).

Level of supplementation is another factor which is determinant of the individual response. Roberts et al. (2009) supplemented cattle grazing annual ryegrass with increasing levels of corn and found that ADG was not increased at supplementation levels of 0.5% BW. Similarly, Latimori et al. (2008) reported no increase in ADG when steers were supplemented with corn at 0.7% BW. We supplemented at 0.67% BW, and that might also be a possible explanation for the lack of effect of supplementation in ADG.

Data from grass chemical composition (Table 3.3) shows that varieties did not differ in WSC, NDF, ADF and CP content ($P > 0.05$). The lack of difference in nutritional quality between varieties would explain the lack of effect of variety on animal performance, which is dependent on both forage nutritive value and forage allowance (Sollenberger and Vanzant, 2011). Theoretically, high sugar cultivars should show a higher concentration of WSC in leaf blades than conventional ones (Edwards et al., 2007).

However, the expression of this difference seems to be dependent on environmental conditions (Parsons et al., 2004, Edwards et al., 2007). Among those conditions, temperature plays an important role. Experiments carried out in New Zealand (Parsons et al., 2004) showed that differences between high sugar and control cultivars were only significant when night temperatures were low (10° C) or when there was a previous phase of cold and short days. Another experiment (Hopkins et al., 2002) compared two night/day temperature regimes (30°C/20°C vs 20°C/7°C) and found that total content and differences between varieties were higher in the 20°C/7°C regime. Rasmussen et al. (2014) analyzed high sugar perennial ryegrass at different temperature regimes (20◦C/20◦C, 20◦C/10◦C, or 10◦C/10◦C, light/dark temperature) and found that WSC content and fructosyltransferases (i.e., the enzyme that catalyzes the polymerization of fructans) activity was higher at low temperature. To the authors' knowledge, our experiment is the first reported experiment of high sugar varieties tested on field conditions on Southeastern US. From the above cited studies, there appears to be a genotype x environment interaction and the high sugar trait does not express equally across all environmental situations (Parsons et al., 2004, Edwards et al., 2007, Rasmussen et al., 2009). We speculate that high temperatures might have impaired expression of the high sugar trait. Further research would be desirable to identify and develop ryegrass varieties with the ability to store higher levels of WSC even in warmer climates (Rasmussen et al., 2014), like the Southeastern US.

Despite numerical differences, no statistical differences were detected for forage intake or DM intake due to supplementation ($P > 0.05$, Table 3.4). This is consistent with the lack of effect of supplementation on individual ADG. In most of the cases, energy

supplementation is expected to reduce forage intake (Bargo et al., 2003, Roberts et al., 2009), but generally the substitution rates are usually lower than 1, leading to an increased total DM intake in supplemented cattle (Waldo, 1986, Bargo et al., 2003). In our case, there were a numerical decreases in forage intake and numerical increases in total DM intake in the supplemented group, but differences were not significant, due to high variability in data.

Similarly, there were no statistical difference ($P > 0.05$) in total DM digestibility due to variety or supplementation (Table 3.4). Similarly, French et al. (2001) did not detect differences in total diet digestibility when supplementing steers at high forage availability with concentrates. In our experiment, the lack of difference in DM digestibility might be reflecting the fact that ryegrass is highly digestible (Morrison, 1980, Frame, 1991), especially under continuous grazing which allow for higher diet selection. Therefore, the expected overall increase in diet quality might have not been as high as expected, because the basal forage quality was high by itself. On the other hand, it is possible that any positive effect of grain supplementation on DM digestibility was counterbalanced by associative effects in which concentrate might have depressed forage fiber digestibility (Vadiveloo and Holmes, 1979, Horn and McCollum, 1987).

CONCLUSION

We did not detect differences in nutritive value or animal performance between high sugar and conventional varieties. No effects of corn supplementation were detected in animal performance, forage intake, DM intake or DM digestibility.

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Table 3.1. Effect of ryegrass variety and cracked corn supplementation on final weight and average daily gain of Angus steers.

	Variety		Corn			*P-Value*		
	Enh	Lone	0%BW	0.67%BW	SEM	Var	Corn	VxC
Initial LW (Kg)	338.8	341	337.7	342	24.47	0.78	0.58	0.98
Final LW (Kg)	455.3	464.3	457.3	462.3	3.98	0.17	0.21	0.16
ADG ($Kg.d^{-1}$)	1.34	1.41	1.35	1.41	0.295	0.26	0.20	0.21

Var= annual ryegrass variety, Enh= Enhancer, high sugar annual ryegrass variety, Lone= Lonestar, conventional annual ryegrass variety, Corn= cracked corn supplementation, VxC= Var x Corn interaction, BW= Body weight, AVG= average

Table 3.2. Dry matter availability (kg DM. ha^{-1}) and forage allowance (kg DM. 100 kg LW^{-1}) for two annual ryegrass varieties

	Variety			*p-value*
	Enh	Lone	SEM	Variety
Availability (Kg DM. ha^{-1})	1615.9	1585.2	563.50	0.90
Allowance (kg DM. 100 kg LW^{-1})	122.4	127.8	31.23	0.74

Enh= Enhancer, high sugar ryegrass variety, Lone= Lonestar, conventional ryegrass variety, SEM= Standard error of the mean,

Table 3.3. Chemical composition of two annual ryegrass varieties

	Variety			*p-value*
	Enhancer	Lonestar	SEM	Variety
OM	90.95	91.51	2.772	0.69
NDF	44.34	44.79	2.955	0.70
ADF	22.11	22.36	1.893	0.79
WSC	20.57	20.71	2.610	0.92
CP	13.85	12.77	2.281	0.37

Enh= Enhancer, high sugar variety, Lone= Lonestar, conventional variety, SEM= Standard error of mean. OM= Organic matter, WSC= Water soluble carbohydrates, CP = crude protein. Data expressed as g. 100 g DM^{-1}

Table 3.4. Forage intake, total DM intake and *in vivo* DM digestibility of diets for Angus steers grazing two varieties of annual ryegrass and two levels of cracked corn supplementation, during year 2

	Variety		Corn			*p-Value*		
	Enh	Lone	0%BW	0.67%BW	SEM	Var	Corn	SxV
Year 2								
Forage intake (kg DM.d^{-1})	5.73	5.04	5.85	4.92	0.537	0.46	0.23	0.60
DM intake (kg DM.d^{-1})	6.78	6.07	5.85	7.00	0.555	0.46	0.18	0.64
DM digestibility (g.100 g DM^{-1})	77.88	73.85	74.59	77.14	3.398	0.49	0.15	0.81

Enh= enhancer, high sugar ryegrass, Lone= lonestar, conventional ryegrass, BW= body weight, SEM= standard error mean, Var= variety, VxC= variety by corn interaction

CHAPTER FOUR

COMPARISON OF FOUR METHODS FOR DETERMINING IN VITRO RUMINAL DIGESTIBILITY OF ANNUAL RYEGRASS

ABSTRACT

Multiple *in vitro* methods to assess forage digestibility have been developed, but little is known regarding apparent dry matter digestibility (DMD) results among them. The objective of this study was to compare three different rumen *in vitro* DMD methods (Daisy^{II} [D], Batch Culture [BC] and the Ankom Gas Production System [G]) at four incubation times ([IT],12, 24, 36 and 48 h). Additionally, results obtained at 24 h were compared with those obtained from dual-flow continuous fermenters [CF]. Annual ryegrass (*Lolium multiflorum*; 91.1% OM, 33.8% NDF, 16.6% crude protein [CP], 27.3 % water soluble carbohydrates) was clipped from an ungrazed pasture, dried (<60 °C, 48 h) and ground in a Wiley mill (1 mm). Three runs of each method were conducted using rumen fluid from a cannulated Holstein cow in mid-lactation fed a 34% corn silage, 6% grass hay and 60% grain TMR. Ankom F57 acetone pre-rinsed bags containing 0.5 ± 0.01 g of sample were used for D, BC and G. Apparent DMD coefficients in CF were estimated in three periods (7d adaptation, 3d collection) ran simultaneously with the other methods. The same buffer was used for all four methods. Data were analyzed using mixed procedure of SAS in a model including method, IT and period as a random factor, with IT as a repeated measure. Means within each IT were compared by PDIFF function. Results indicated that D predicted resulted in higher DMD than G and BC at IT greater than 12 h.

Apparent DMD estimated using CF was similar to the obtained with BC and G at 24 h, but lower than D. We conclude that when utilizing different *in vitro* digestibility methods results may differ, and caution should be exercised when comparing digestibility data obtained by different methods.

INTRODUCTION

Although *in vivo* determinations remain as the golden standard for digestibility assessment, they are expensive and labor demanding. Therefore, the development and use of *in vitro* techniques are becoming widely used (Holden et al., 1999). Over the years, multiple *in vitro* methods have been developed, but information regarding their compatibility is scarce. The *in vitro* batch culture (BC, Tilley and Terry, 1963) consisted of a digestion in a glass tube containing buffer and rumen fluid incubated at 38ºC. The DAISY^{II} (D, Ankom Technology, Fairport, NY) is a widely used method which allows simultaneous analysis of multiple samples. This system consists of an incubator at 39.5 ºC where four glass jars are incubated, allowing up to 96 samples per run (Robinson et al., 1999). The amount of gas produced during fermentation has also been used to estimate digestibility. Menke and Steingass (1988) proposed to use gas volume and feed composition data to estimate energy content of feeds. Recently, automated and standardized methods for measuring gas pressure have been developed, like the Ankom RF Gas Production System (G; Ankom Technology Corporation, Fairport, NY). This system measures gas pressure at time intervals, yielding results of total digestibility as well as kinetics of digestion. Finally, rumen continuous fermenters (CF) are reaction vessels where

rumen fermentation runs continuously for longer periods than in the aforementioned methods. This design closely mimics ruminal conditions and allows for natural stratification of feed particles, temperature compensation, and salivary buffering, similar to what occurs in the rumen.

These methods are commonly used to estimate and report *in vitro* apparent dry matter digestibility (DMD). Although comparisons between some techniques are available (Holden et al., 1999, Wilman and Adesogan, 2000), comparisons of the 4 techniques including CF are limited. Therefore, an experiment was conducted with the objective of comparing these methods to estimate rumen DMD (D, BC, G and CF). Our hypothesis was that these *in vitro* methods yield different results, especially at longer incubation times (IT).

MATERIAL AND METHODS

The experimental design was a randomized complete block design. The treatments consisted of the *in vitro* DMD methods (D, BC, G and CF). The experiment consisted of three periods in which all the methods were ran simultaneously. Rumen fluid was collected from a cannulated Holstein dairy cow in mid-lactation fed a TMR diet (34% corn silage, 6% grass hay and 60% grain-mix). All surgical and animal care protocols were approved by the Clemson University Animal Care and Use Committee. All methods were evaluated using annual ryegrass (*Lolium multiflorum,* var. Enhancer, Sucraseed, Tangent, OR) harvested from an ungrazed pasture in vegetative stage. The grass was cooled immediately after harvest and later dried at 60 ºC until constant weight. Dry samples were ground to

pass a 1-mm sieve of a Wiley mill (Arthur H. Thomas, PA). Chemical analysis included NDF and ADF determination (Van Soest et al., 1991), ash content by combustion in muffle furnace (600ºC, 2 h), total N by combustion using a Leco CNS Analyzer (Leco Corp., St. Joseph, MI), and water soluble carbohydrate determination using the phenol and sulfuric acid colorimetric technique (Hall, 2013). Rumen contents were collected from five areas of the rumen (cranial, caudal, dorsal, ventral, and central) and contents were homogenized in a preheated blender while purged with CO_2. A buffer composed by $NaH_2PO_4*H_2O$ (5.8 g/l), NaCl (0.28 g/l), $CaCl_2$ (0.02 g/l), MgCl (0.04 g/l), Urea (0.3 g/l) and $NaHCO_3$ (3.67 g/l) was used for all the methods (Slyter et al., 1966). For the methods D, BC and G, acetone-rinsed Ankom F57 bags were filled with 0.5±0.05 g of ryegrass and heat sealed. The use of Ankom F57 fermentation bags for BC is a modification from technique originally published by Tilley and Terry (1963). We used these to make the methods more comparable, since low dry matter recovery from Erlenmeyer flasks through filtration has previously been reported (Hall and Mertens, 2008). The blended rumen fluid was filtered through a two layers cheesecloth and mixed with the pre-warmed buffer at a 1:4 rumen fluid:buffer ratio, except for the CF, in which the rumen fluid:buffer ratio was 1:1 (Jenkins et al., 2014). For D, BC and G, four IT were assessed: 12, 24, 36 and 48 h. In the case of CF, DMD was compared to those obtained in the other methods at IT 24 h. In D, a total of 20 bags per run and IT were assessed. Briefly, this system consists of an incubator at 39.5 ºC where four glass jars rotate by means of gear drives (Robinson et al., 1999). To evaluate each IT, the jar was quickly removed from the incubator when the time was reached. In the case of BC, filter bags containing 0.5±0.05 g of sample were placed into 250 ml Erlenmeyer

flask containing 125 ml of the mix of rumen fluid and buffer, and incubated in shaking water bath (39.5 ºC, 40 oscillations per min) during the same IT as in D. Four flasks per incubation time were ran and when IT was reached the four flasks were removed at once. At the end of each incubation period, bags were removed and rinsed under cold tap water until water ran clear, then dried at <60º C for 48 h. In the case of G, the Ankom RF Gas Production System allows for constant estimation, so the record of pressure produced at 12, 24, 36 and 48 h IT were considered. A total of 10 bottles (1 for blank, 1 for standards and 8 for samples) were ran in parallel in each period. Fermentation gases produce pressure changes in the bottle headspace, which are transmitted every 5 min. The readings at each incubation time were cumulated (ΔP) and converted into volume units (GP, ml) by means of the ideal gas law:

$$GP = (\Delta P/Po) \times Vo$$

where ΔP is the cumulated pressure change, Vo is the bottle headspace volume (190 ml), and Po is the atmospheric pressure. Blank bottles were used to adjust the baseline by subtraction. Then, DMD was estimated using the Menke Equation (Menke et al., 1979), as follows:

$$DMD = 14.88 + 0.889\ GP + 0.45\ CP + 0.0651\ Ash$$

where DMD is apparent dry matter digestibility, GP is gas production (ml), CP and Ash are the crude protein and ash content of the grass, respectively.

The dual-flow CF used in the experiment is a modified version of the design described by Teather and Sauer (1988), the main modifications being an overflow sidearm angled downward at 45º and a faster stirring rate (45 rpm). Therefore, particles stratify into an upper mat, a middle liquid layer of small feed particles, and a lower layer of dense particles. Solid passage rate was fixed at 5%.h^{-1} and liquid dilution rate at 12%.$h^{-1,}$ by regulating the buffer infusion pumps at 90 ml.h^{-1}. Fermenters were fed 30 g DM per day, in two daily feedings at 0800 and 1600 (15 g DM.d^{-1} each). Buffer pH was controlled by adjusting with 6N NaOH and rumen fluid pH was measured twice a day coinciding with feeding time to keep it above 6.8 before morning feeding. The temperature was kept at 39.5°C by a circulating heated water bath (Julabo, PA, USA). Fermenters were constantly purged with CO_2 (20 ml/min) to preserve anaerobiosis. Periods consisted of 7 d of adaptation and 3 d of sample collection, during which total volume and DM content of overflow was measured. DMD was calculated by difference between the amount DM fed daily and the DM collected from the outflow.

Data were analyzed using mixed procedure of SAS in a model including DMD method, IT and their interaction as fixed factors and period as a random factor, with IT as repeated measures. Denominator degrees of freedom were calculated using the Kenward-Roger method, and a first-order autoregressive covariance-structure was used. Means within each incubation time were compared by PDIFF function. Results obtained at 24 h IT were compared including CF, using mixed procedure of SAS in a model that included period and method.

RESULTS AND DISCUSSION

Ryegrass chemical composition is shown in Table 4.1. No difference between methods was observed at 12 h IT ($P > 0.05$), but for 24, 36 and 48 h, D showed higher ($P < 0.05$) DMD than BC and G (Figure 4.1). Dry matter digestibility estimated using CF was similar to that obtained with BC and G at 24 h, but lower than the estimated with D (Figure 4.1). Holden (1999) compared D with a version of BC slightly modified from the original Tilley and Terry (1963) method for ten different feeds and found no effect of method on DMD. However, some important differences exist between Holden (1999) experiment and our experiment. In BC, we used Ankom F57 bags, whereas Holden (1999) made it with the sample immersed directly in the inoculum, filtering later with filter paper. Also, Holden (1999) incubated the tubes in a water bath and swirled them by hand periodically, while we used a water bath with a shaker at 40 rpm. These are physical factors which could have an effect, leading to the differences found in our experiment. For example, agitation and compression due to movements have been suggested as important factors in *in vitro* DMD assessment (Marinucci et al., 1992, Hall and Mertens, 2008). Daisy jars rotate constantly (0.95 rpm) and they possess an internal septum with fenestrations which leads to the complete immersion of the bags in the inoculum in every spin of the jar, whereas in BC or G, once the fermentation starts, gases start to accumulate within the bag, thus expanding it and making it float. Additionally, the shaking effect at 40 rpm is less than that of the rotation of D. Marinucci et al. (1992) compared *in vitro* DMD of samples contained in

synthetic fiber bags versus samples free in the inoculum. They reported that bags were expanded with gas, which affected the flow of inoculum and microorganisms into the bag. These results are consistent with those reported in the present experiment. DMD was consistently higher for samples floating freely in the flask, which could explain any differences with the Holden (1999) method. However, the use of filter bags may be advantageous, since filtration and recovery have been mentioned as sources of inconsistency in digestibility coefficients (Robertson et al., 1972, Hall and Mertens, 2008). Additionally, Hall and Mertens (2008) reported that vertically positioned tubes rendered lower digestibilities than horizontally placed ones. This is also a difference between D, in which flasks rest almost horizontal (Robinson et al., 1999), and BC and G, in which Erlenmeyer or flasks are placed vertically.

Dry matter digestibility results obtained by BC and G were similar at all IT. As previously mentioned, fermentation conditions in terms of source of inoculum, buffer, temperature, shaking conditions, positioning and use of bags were identical between these two methods. Therefore, it can be said that DMD estimated through G using the Menke equation (Menke et al., 1979) was acceptable and comparable with that obtained using a traditional method as BC.

Despite the fact that in CF substrate is directly immersed into the inoculum, results coincided with BC and G at 24 h IT. To our knowledge, this is the first comparison of DMD assessed with this kind of CF to other *in vitro* techniques. Continuous fermenters allow for a constant escape of solid particles, which in our case was set at 5%/h. Then, at

least a portion of potentially digestible DM escapes without being digested, and DMD in CF is dependent on solid dilution rate, with higher dilution rates yielding lower digestibilities (Hoover et al., 1982, Qiu et al., 2004). Stirring speed is also an important factor affecting final DMD. This phenomena does not occur with the other digestibility methods, in which no escape of solids is possible. Therefore, when comparing DMD results obtained in CF with other methods, solid dilution rate and stirring speed, which in turn determine the retention time and time of exposure of feed to microorganism, should be taken into account (Crawford et al., 1980, Hoover et al., 1982). It is interesting that DMD data obtained with CF was close to the values obtained for the methods at IT of 24 h, since in the CF overflow volume and samples are taken after a period of 24 h of fermentation.

CONCLUSION

We conclude that, under the conditions of this experiment, different *in vitro* digestibility methods yield different results. At 24 h of incubation, G and BC agreed with the results obtained in CF. Similar results were observed for BC and G at all IT, whereas D gave a higher DMD at incubation times longer than 12 hours. Even though these differences among methods do not impede ranking of feedstuffs on the basis of DMD within method, caution should be exercised when comparing *in vitro* DMD data obtained by different methods.

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Table 4.1. Chemical composition of annual ryegrass sample used for the comparison of the four in vitro digestibility methods

Composition	(g .100g^{-1} DM)
Organic matter	91.10
Neutral detergent fiber	33.80
Acid detergent fiber	17.70
Water Soluble Carbohydrates	27.30
Crude Protein	16.60

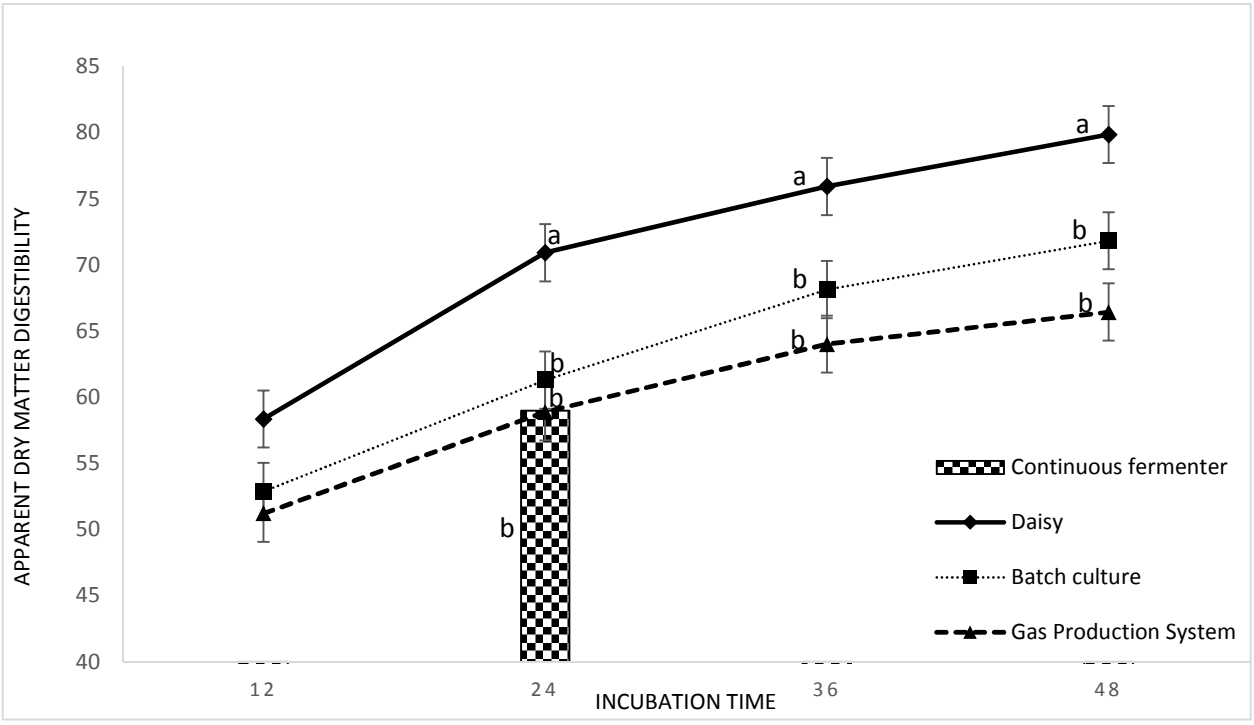

Figure 4.1. Percentage of dry matter disappearance of annual ryegrass samples assessed by four in vitro digestibility methods at four incubation times (12, 24, 36 and 48 h). Different letters indicate differences due to method within incubation times ($P < 0.05$). Error bars = 2.1, Standard error of the mean.

CHAPTER FIVE

EFFECTS OF WATER SOLUBLE CARBOHYDRATE AND SOLUBLE PROTEIN LEVEL ON MICROBIAL PROTEIN SYNTHESIS, NUTRIENT DIGESTIBILITY AND FERMENTATION PARAMETERS IN CONTINUOUS CULTURE FERMENTERS

ABSTRACT

Nutrient synchrony is considered an important factor affecting the ability of microbes to synthetize microbial protein. The objective of this experiment was to evaluate the effects of two different levels of water soluble carbohydrates (WSC) and three levels of soluble protein (SP) in annual ryegrass on microbial protein synthesis, fermentation profile and nutrient digestibility, using continuous culture fermenters. Six fermenters were used on a 3 x 2 factorial arrangement with three levels of WSC (21, 24 and 27 g.100 g DM^{-1}; LWSC, MWSC and HWSC, respectively) and two levels of SP (5.2 and 9.4 g.100 g DM^{-1}, LSP and HSP, respectively). Four periods of 10 days were ran sequentially, with the first 7 days for diet adaptation and 3 days for sampling and measurements. Microbial protein synthesis was assessed using the purine to nitrogen ratio as marker. Higher levels of WSC led to a higher microbial protein synthesis when expressed as g bacterial crude protein (CP) per day, but no differences were found when analyzed as g bacterial CP per 100 g digested DM. No differences in microbial protein synthesis were detected due to SP level; however, there was a significant interaction between WSC and SP level in microbial protein

synthesis. Water soluble carbohydrates level did not affect fermentation pH, ammonia concentration or total volatile fatty acids concentration ($P > 0.05$), whereas higher levels of SP increased ammonia concentration ($P < 0.0001$) and tended to increase pH at 1 and 2 hours postfeeding. Higher SP levels also increased acetic proportion ($P = 0.01$) and tended to increase acetic:propionic ratio ($P = 0.06$). Dry matter, NDF and ADF digestibility were not affected by treatments. It is concluded that WSC level in ryegrass samples increased microbial protein synthesis and that SP and WSC level might have a positive interaction at limiting levels of ammonia in the culture.

INTRODUCTION

Rumen microbes have the ability to synthetize microbial protein from non-protein nitrogen sources, provided that they have a source of carbon skeletons to form amino acids. However, it has been suggested that they not only would need an adequate provision of nitrogen and carbon skeletons in terms of quantity, but also in terms of synchrony, implying that nutrients should be available at the same time in the proportions needed by microorganisms (Johnson, 1976, Kim et al., 1999, Hall and Huntington, 2008). High quality grasses, like annual ryegrass and other winter annuals, often show high crude protein (CP) concentrations, mainly in the form of rapidly degradable and highly soluble non-protein nitrogen (Kinston-Smith and Theodorou, 2000, Cosgrove et al., 2007). This in turn leads to ammonia buildup in the rumen, which microorganisms cannot efficiently capture (Stern et al., 1978). Therefore, ammonia must be absorbed, detoxified in the liver

and secreted through urine, in a process that implies nitrogen use inefficiency and that has negative environmental implications (Edwards et al., 2007, Da Silva et al., 2014).

Temperate grasses accumulate energy surpluses from photosynthesis in the form of highly soluble carbohydrates (i.e., fructans), commonly called water soluble carbohydrates (WSC) or simply "sugars". This fraction of the organic matter is readily available to microorganisms immediately after entering into the rumen (Johnson, 1976); therefore, they provide carbon skeletons which are synchronized in their release with highly soluble protein (SP). Therefore, an increased supply of WSC in diets high in non-protein nitrogen could lead to more efficient microbial nitrogen use and to an increased microbial protein synthesis (Mansfield et al., 1994).

In vivo systems are exceedingly complex and multiple interactions and homeostatic mechanisms might act together to counterbalance the effect of nutrient asynchrony, reducing therefore the impact of nutrient synchrony on animal performance or microbial protein synthesis (Hall and Huntington, 2008). On the other hand, *in vitro* systems, like continuous culture fermenters, might be more sensitive to changes in the relative supply of nutrients, helping to elucidate the real impact of nutrient synchrony on ruminal environment and microbial protein synthesis. The objective of this experiment was to evaluate the effect of two levels of total crude protein and SP and three levels of WSC in annual ryegrass samples on microbial protein synthesis, fermentation profile and nutrient digestibility, using continuous culture fermenters. Our hypothesis was that higher levels of WSC should lead to increased microbial protein synthesis, reducing the ammonia increase after feeding high SP diets.

MATERIAL AND METHODS

Treatments. Six treatments were generated by a 3 x 2 factorial combination of 3 levels of WSC (21, 24 and 27 g.100 g DM^{-1}, ; LWSC, MWSC and HWSC, low, medium and high WSC, respectively) and 2 levels of soluble protein ([SP], 5.2 and 9.4 g.100 g DM^{-1}, LSP and HSP, low and high SP, respectively). A sample of dry and ground annual ryegrass (*Lolium multiflorum*, Lam.) was used as the basic feed to which crystalline fructose (Tate & Lyle, IL, US, 99% purity) and ground urea (460 mg nitrogen/ g urea) were added to obtain the different levels of WSC and SP content. Nutritional composition of treatments can be found in Table 5.1. Feed analysis included determinations of NDF (Van Soest et al., 1991), ADF (AOAC, 2000), ADL (Goering and Van Soest, 1970), CP (AOAC, 2000) soluble protein (Krishnamoorthy et al., 1982), degradable protein (Krishnamoorthy et al., 1983), WSC (Dubois et al., 1956, Hall, 2013), ether extract (AOAC, 2000) and starch (Hall, 2009) content.

Four periods of 10 days were ran sequentially, with the first 7 days for diet adaptation and 3 days for sampling and measurements.

Continuous culture fermenters. The dual-flow continuous culture fermenters used in this experiment are a modified version of the design described by Teather and Sauer (1988), the main modifications being an overflow sidearm angled downward at 45º, to facilitate emptying, and a faster stirring rate (45 rpm). These devices allow for particle stratification into an upper mat of solid floating material, a middle layer of small feed

particles, and a lower layer of dense particles (Lee and Jenkins, 2011). Solid passage rate was fixed at 5 %.h^{-1} and liquid dilution rate at 12%.h^{-1}, by regulating the buffer infusion pumps at 90 ml.h^{-1}.

At the beginning of each period, whole rumen contents were taken from two cannulated Holstein dairy cows in mid-lactation fed a totally mixed ration comprised of 34% corn silage, 6% grass hay and 60% grain-mix diet. All surgical and animal care protocols were approved by the Clemson University Animal Care and Use Committee. Liquid and solid rumen contents were collected from five areas of the rumen, transported in vacuum containers to the lab and homogenized in a preheated blender while purged with CO_2. Then, the blended mix was filtered through a double layer cheesecloth and mixed with the buffer at a 1:1 ratio and added into the continuous culture fermenter vessel (approximately 750 ml total volume). The buffer was composed by $NaH_2PO_4*H_2O$ (5.8 g/l), NaCl (0.28 g/l) , $CaCl_2$ (0.02 g/l), MgCl (0.04 g/l), Urea (0.3 g/l) and $NaHCO_3$ (3.67 g/l) (Slyter et al., 1966). The temperature was kept at 39.5°C by a circulating heated water bath (Julabo, PA, USA). Fermenters were constantly purged with CO_2 (20 ml/min) and rubber sealed to preserve anaerobiosis. Fermenters were fed 40 g DM of the respective treatment per day, in two daily equal portions (0800 and 1600, 20 g DM each time).

Sample collection and measurements. Measurements were recorded during the last three days of each period. During these days, overflow volume was recorded daily in individual vessels kept on ice to stop organic matter fermentation. After measuring

volume, the material was placed in a beaker under stirring and a 10 ml sample of overflow was taken from it with wide mouth a pipette (0.8 mm). This sample was used to estimate overflow DM content. Additionally, samples of overflow (1000 $ml.d^{-1}$) were kept to estimate NDF and ADF content, as well as to estimate the nitrogen to purine ratio and, from that, microbial protein synthesis. Dry matter, NDF and ADF digestibility coefficientes were estimated from the difference between the amount of each nutrient fed daily and the amount collected from the outflow. NDF and ADF content of the overflow were estimated in an ANKOM 2000 analyzer (Van Soest et al., 1991). Every morning during the sampling period, overflow samples were taken and treated to isolate a bacterial pellet in which to estimate the nitrogen to purine ratio. The overflow was treated by differential centrifugation, which consisted in two consecutive centrifugations at 500 x g for 20 and 10 minutes to separate and discard solid feed particles. The supernatant was saved in each of these centrifugations and was later treated with two centrifugations at 8000xg during 20 minutes, washing with PBS and distilled water. Purine content in bacterial pellet and overflow was determined according to Zinn and Owens (1986).

Samples from the culture were taken during the last day of each period at -2, 0, 2, 4, 6 and 8 h, being 0 the first daily feeding time at 0800, and 8 the last daily feeding at 1600. Measurements of pH were done at the same sampling times (plus an additional measurement at 1 h) and these measurements were used to estimate average pH. A 10 ml sample was taken from the culture with a wide mouth pipette (0.8mm) and put into a beaker under stirring, from which a sample of 4 ml of fluid was taken and conserved into polycarbonate tube containing with 1 ml of 25% (w/w) metaphosphoric acid, which was

later frozen. After thawing, samples were centrifuged first at 4000 g during 10 min and a 2 ml aliquot was pipetted from the supernatant and centrifuged again at 16000 g during 15 min. These samples were used ammonia (NH_4^+) and VFA concentration assessment. Ammonia concentration was estimated by colorimetric technique (Chaney and Marbach, 1962). Briefly, the sample is treated with a solution containing phenol, sodium nitroferricyanide (50 mg/l) and sodium tungstate (8.25 g/l) and then with another solution containing anhydrous disodium phosphate (Na_2HPO_4) and sodium hypochlorite. The mix is then incubated at 65ºC for 30 min and a blue colorimetric reaction occurs, which can be then read at 625 nm in the spectrophotometer.

In the VFA samples, 1 ml of supernatant was combined with 100 µl of 2-ethylbutyric acid (86µmol/100µl) as internal standard, and samples were analyzed by gas chromatography with flame ionization detector on a Zebron ZB-FFAP 30 m x 0.25 mm x 0.25µm column (Phenomenex, Torrance, CA). The injection volume was 0.1 µl and samples were injected with a split ratio 0f 10:1. Injector was kept at 270 ° C and detector at 250 ° C. The carrier gas was hydrogen at a flow rate of 26.9 ml/min. Column oven temperature was programmed to increase from 120 to 150 ° C at a rate of 12 °C/min, and from 150 to 220 ° C at a rate of 20° C/min. Standard curves were ran for each of the VFA using a standard VFA mix (Sigma-Aldrich VFA mix, PA, US) to estimate the total VFA concentration (mM) as well as the molar proportion of the individual VFA.

During the 7-d adaptation period, daily total overflow and medium pH was measured twice a day during the 10 days of each period, to keep a control of buffer flow and fermentation.

Statistical analyses. Dry matter, NDF and ADF digestibility, and microbial protein synthesis data were analyzed using the mixed procedure of SAS (SAS Inst., Inc., Cary, NC) based in the following model: $Y_{ijk}= \mu + \gamma_i + \pi_j + \gamma\pi_{ij} + \rho_k + \varepsilon_{ijk}$, where Y_{ijk} is the observed value, μ is the overall mean, γ_i is the water soluble carbohydrate effect (i= 1 to 3), π_j is the soluble protein effect (j= 1 to 2), $\gamma\pi_{ij}$ is the interaction between water soluble carbohydrate and soluble protein, ρ_k is the random effect of period (k = 1 to 4) and ε_{ijk} is the experimental error. Ammonia, VFA, and pH data were analyzed with repeated measures using the mixed procedure of SAS (SAS Inst., Inc., Cary, NC) based in the following model: $Y_{ijkm}= \mu + \gamma_i + \pi_j + \gamma\pi_{ij} + \rho_k + \gamma\pi\rho_{ijk} + \delta_m + \gamma\delta_{im} + \pi\delta_{jm} + \gamma\pi\delta_{ijm} + \varepsilon_{ijkm}$, where Y_{ijkm} is the observed value, μ is the overall mean, γ_i is the water soluble carbohydrate effect (i= 1 to 3), π_j is the soluble protein effect (j= 1 to 2), $\gamma\pi_{ij}$ is the interaction between water soluble carbohydrate and soluble protein, ρ_k is the random effect of period (k = 1 to 4), $\gamma\pi\rho_{ijk}$ is the random interaction between water soluble carbohydrate, soluble protein, and period, δ_m is the sampling hour effect (m = 1 to 6), $\gamma\delta_{im}$ is the interaction between water soluble carbohydrate and sampling hour, $\pi\delta_{jm}$ is the interaction between soluble protein and sampling hour, $\gamma\pi\delta_{ijm}$ is the interaction between water soluble carbohydrate, soluble protein, and sampling hour, and ε_{ijk} is the experimental error. Least square means were generated and separated by Fisher's protected LSD. Significance was determined at $P < 0.05$. Differences of $P > 0.05$ and $P < 0.10$ are discussed as trends.

RESULTS AND DISCUSSION

Fermentation parameters and nutrient digestibility. There was not effect of WSC or SP on pH ($P > 0.05$, figure 5.1). There was significant effect of sampling hour ($P < 0.0001$), with lower pH following feeding, reaching its lower value at 2h postfeeding (figure 1). There was no significant interaction between WSC and SP level, or between WSC and sampling hour ($P > 0.05$). However, there was a significant interaction between SP and sampling hour ($P = 0.003$). At hour 1 and 2 post feeding, there was a higher pH in HSP. At hour 1 post feeding, pH was 6.42 and 6.26 in HSP and LSP, respectively ($P = 0.04$, data not shown), whereas at hour 2 post feeding pH was 6.18 and 5.98 in HSP and LSP, respectively ($P = 0.02$, data not shown). No differences were detected at the rest of the sampling hours. Since the differences in the treatments SP contents were obtained by the addition of urea, it seems that the urea had a buffer effect, acting like an alkali and increasing pH. Once into the rumen, urea is rapidly converted into ammonia by microorganisms' ureases. Ammonia has a pKa value of 9.21 and therefore at rumen pH it is highly ionized as $NH4^{+}$. Its ability to bind protons makes it an important pH regulator (Dijkstra et al, 2012).

Higher levels of SP produced a significantly higher ammonia concentration ($P < 0.0001$, figure 2), whereas the main effects of WSC or the interaction of WSC x SP were not significant ($P > 0.05$). This implies that higher WSC level had no effect attenuating the increase in ammonia concentration. This result contrasts with the findings of Kim et al. (1999), who reported that ruminal infusion of maltodextrin reduced rumen ammonia concentration and reduced the peak of ammonia concentration immediately after feeding.

There was also significant effect of sampling hour and a significant interaction between sampling hour and SP level ($P < 0.05$, figure 5.2). Even though the ammonia levels were different between levels of SP at every sampling hour, the differences became more pronounced right after the morning feeding, decreasing gradually with time (Figure 5.2). As previously mentioned, urea is quickly converted into ammonia once it is available for rumen microbes, thus explaining the sharp increase in ammonia concentration after morning feeding. Similar effects of urea addition on ammonia concentration have been reported by Stern et al. (1978).

With respect to VFA, WSC tended to have a significant effect on total VFA concentration ($P = 0.08$, figure 5.3). The total VFA concentration was higher in MWSC. Comparing figures 5.1 and 5.3, it is clear that the lower pH values were detected at sampling hours in which the higher total VFA concentration was measured. Rumen pH and VFA concentration are negatively correlated (Dijkstra et al, 2012). However, in *in vivo* experiment this is partially attenuated by VFA removal through absorption, given that at lower pH, a higher proportion of VFA is at a non-dissociated state, hastening thus the passage through rumen wall by passive absorption (Kohn and Dunlap, 1998). In the case of continuous culture fermenters, where absorption process do not occur, the VFA concentration may be more closely related to pH.

Acetic acid molar proportion (mM. 100 mM^{-1}) was not affected by WSC ($P = 0.15$ Table 5.2), but it was affected by SP ($P = 0.01$) level. The proportion of acetic acid was higher in HSP than in LSP, probably due to the effect of higher levels of SP on pH. Fermentation pH has an impact on the type of VFA produced. As the pH decreases, there

is a shift from the production of acetic towards the production of propionic acid (Bannink et al., 2008). On the other hand, SP level also decreased the molar proportion butyric acid, which was lower in HSP ($P = 0.04$, Table 5.2).

No effects of treatments were found for DM, NDF or ADF digestibilities ($P > 0.05$, Table 5.3). Coincidently, Mansfield et al. (1994) did not find significant effects of altering the non-structural carbohydrates proportion on DM digestibility. With respect to the fiber, the effect of feeding highly fermentable carbohydrates on its digestion has been studied previously (Calsamiglia et al., 2008) and it seems clear that those effects are mediated by the rumen pH, which is one of the most important factors affecting fibrolytic bacteria activity (Russell and Wilson, 1996). Several researchers have shown that fiber digestibility results impaired when average pH is below 6.0 (Mouriño et al., 2001, Calsamiglia et al., 2008, Disjktra et al., 2012). Our average pH values were above 6.0 in all the treatments, which implies that the fermentation environment was apt for a good fiber fermentation even at the higher levels of WSC, and would explain the lack of differences among treatments. On the other hand, the lack of effect of urea addition on DM and ADF digestibility coincides with the report of Stern et al. (1978).

Microbial protein synthesis. There was a significant effect of WSC level on microbial protein synthesis when expressed as g of bacterial CP synthetized per day ($P < 0.0001$, Table 5.4) but not when it was expressed a g of bacterial CP per 100 g of digested DM ($P = 0.58$, Table 5.4). The effects of higher levels of non-structural carbohydrates on

microbial growth and protein synthesis has previously been reported by Stern et al. (1978) and Kim et al (1999). Hennings et al. (1991) found that a pulse dose of WSC at feeding time was the most effective way to increase microbial growth in batch culture. Berthiaume et al. (2010) reported that high WSC alfalfa varieties increased the efficiency of nitrogen use by bacteria, even though no differences in microbial protein synthesis were detected. Water soluble carbohydrates (i.e., fructans and fructose) go quickly into solution once in the rumen environment and would therefore be available for their fermentation, giving ATP and VFA as a by-product that can later be used in combination with nitrogen sources in the synthesis of microbial protein (Johnson, 1976).

Whether to consider g bacteria CP synthetized per day or g of bacterial CP per 100 g of digested DM as the valid indicator of microbial protein synthesis is a point that has been previously discussed by Stern et al. (1978). In our case, it is important to point out that all treatments had exactly the same DM intake (40 g DM. d^{-1}), which was fixed during the experiment design. Therefore, we think it is valid to consider the amount of bacterial CP synthesis per day as a valid indicator of microbial protein synthesis, especially since, even though not significantly different (Table 5.3), numerical differences existed in DM digestibility.

Microbial protein synthesis was not affected by the main effect of SP ($P = 0.39$). Similar to our findings, Stern et al. (1978) did not find effects of higher SP on microbial protein synthesis in high starch diets. It has been suggested that increasing ruminal nitrogen supply when ammonia concentrations are above 5 mg.100 ml^{-1} does not lead to increased microbial protein synthesis (Satter and Slyter, 1974). Our ammonia

concentration values, even in the low SP level, were slightly above this threshold (6.12 mg.100 ml^{-1}). However, even though the main effect of SP level was not significant, there was a significant interaction among SP and WSC level on microbial protein synthesis (Table 5.4). Higher microbial protein synthesis was found when WSC and SP levels were higher, and within the higher level of SP, increasing the level of WSC led to increased microbial protein synthesis (Table 5.4). It is possible that at the lower level of SP, ammonia concentration became limiting for further increase in microbial protein synthesis despite higher WSC supply, since, as aforementioned, the levels of ammonia were close to the threshold suggested by Satter and Slyter (1974). This would explain the differences in our findings and those of Mansfield et al. (1994) or Henning et al. (1991), who did not find significant interactions between non-fibrous carbohydrates level and degradable protein intake. In their case, the report shows that ammonia levels in fermentation culture was not limiting even at the lowest levels of degradable protein intake, whereas in our case we were in practical terms right in the threshold stated by Satter and Slyter (1974). From the comparison of our findings with those of Mansfield et al (1994) and Henning (1991), it might be suggested that SP and WSC level might not show significant interactions when ammonia levels are above the minimum required for a maximal microbial protein synthesis, but the interaction might be significant when the lower levels of SP become limiting. It also should be considered that *in vivo* systems (i.e., live animals) have mechanisms for nitrogen recirculation through saliva (Hall and Huntington, 2008), whereas *in vitro* systems (i.e., continuous culture fermenters) lack this property.

CONCLUSION

Higher levels of WSC led to higher microbial protein synthesis. There was also a significant interaction between WSC and SP level in microbial protein synthesis, which could be reflecting that at the lowest level of SP, low levels of ammonia were limiting microbial growth. Higher levels of SP increased ammonia concentration and tended to increase pH immediately post-feeding. This in turn led to higher acetic proportion and increased acetic:propionic ratio.

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Table 5.1. Chemical composition of diets differing in water soluble carbohydrates and soluble protein content, fed to continuous culture fermenters.

	Water soluble carbohydrates					
	LWSC		MWSC		HWSC	
SP content	LSP	HSP	LSP	HSP	LSP	HSP
*Item**						
CP	14.4	18.7	15.0	18.0	14.5	19.1
SP	5.2	9.3	5.4	9.4	5.0	9.6
RDP	9.8	14.0	10.2	13.7	9.8	14.5
NDF	49.2	49.0	47.4	46.5	45.4	46.1
ADF	30.2	29.5	29.3	29.2	28.9	30.8
ADL	2.9	4.2	2.5	2.9	2.5	2.4
WSC	21.2	21.0	24.1	24.5	27.1	26.9
Ether extract	2.8	1.9	2.8	2.3	2.7	2.2
Starch	2.3	2.2	1.3	2.0	1.1	1.0

Item:* expressed as g. 100 g DM^{-1}.

Water soluble carbohydrates: Low= 21 g.100 g DM^{-1} , Medium: 24 g. 100 g DM^{-1} , High 27 g. 100 g DM^{-1} ; SP (soluble protein) content: SPLow: 5.2 g.100g $DM^{-1,}$ SPHigh: 9.4 g.100g DM^{-1}. CP= crude protein, SP= soluble protein, RDP= Ruminally degradable protein, NDF= neutral detergent fiber, ADF: acid detergent fiber, ADL: acid detergent lignin, WSC: water soluble carbohydrates, EE: ether extract.

Table 5.2. Individual volatile fatty acids concentration of diets differing in water soluble carbohydrates and soluble protein content at -2, 0, 2, 4, 6 and 8 h post feeding, fed to continuous culture fermenters.

	LWSC		MWSC		HWSC		SEM	*p- value*						
VFA (mM/100 mM)	LSP	HSP	LSP	HSP	LSP	HSP		WSC	SP	WSCxSP	H	WSCxH	SP x H	WSCxSPxH
Acetic							1.416	0.15	0.01	0.93	<0.0001	0.07	0.26	0.97
-2 h	49.20	53.28	45.77	49.85	46.90	51.84								
0 h	50.72	53.15	47.08	50.50	48.14	51.84								
2 h	52.06	54.38	46.56	49.40	47.80	50.91								
4 h	45.12	51.05	42.80	46.18	41.47	47.51								
6 h	46.17	50.23	44.85	48.17	43.20	47.43								
8 h	47.46	52.28	47.44	50.33	45.58	50.52								
Propionic							1.201	0.58	0.24	0.65	0.001	0.21	0.82	0.96
-2 h	24.11	23.78	26.55	25.04	26.56	23.97								
0 h	23.96	23.84	26.66	25.26	26.51	24.18								
2 h	24.83	25.04	26.12	26.18	27.61	24.81								
4 h	26.51	26.01	26.05	26.11	27.83	25.93								
6 h	25.45	25.03	25.69	25.37	27.13	24.81								
8 h	25.41	24.45	25.82	24.72	27.36	24.98								
Butyric							0.830	0.66	0.04	0.47	<0.0001	0.04	0.08	1.00
-2 h	16.74	14.87	16.75	15.19	15.16	15.54								
0 h	15.67	14.41	15.55	14.42	14.45	14.70								
2 h	15.19	13.76	17.74	16.21	15.48	15.97								
4 h	19.63	16.21	21.19	18.36	20.27	17.88								
6 h	19.13	17.36	18.83	17.25	18.66	18.57								
8 h	17.96	16.07	16.90	15.65	16.91	16.45								
Ac:Prop ratio							0.134	0.35	0.06	0.72	<0.0001	0.09	0.51	0.95
-2 h	2.06	2.26	1.74	1.99	1.75	2.20								
0 h	2.12	2.24	1.78	2.00	1.80	2.17								
2 h	2.11	2.17	1.80	1.89	1.72	2.08								
4 h	1.70	1.98	1.66	1.77	1.48	1.88								
6 h	1.81	2.02	1.76	1.90	1.58	1.96								
8 h	1.87	2.15	1.85	2.04	1.65	2.07								

LWSC, MWSC and HWSC, low, medium and high water soluble carbohydrates, 21, 24 and 27 g WSC. 100 g DM^{-1}. LSP and HSP, low and high soluble protein, 5.2 and 9.4 g.100 g DM^{-1}. H=sampling hour. SEM= Standard error mean

Table 5.3. Dry matter, NDF and ADF digestibility of diets differing in water soluble carbohydrates and soluble protein content, fed to continuous culture fermenters.

	Water soluble carbohydrates									
	LWSC		MWSC		HWSC		SEM	*p- value*		
SP content	LSP	HSP	LSP	HSP	LSP	HSP		WSC	SP	WSC x SP
*Item**										
DM digestibility	46.8	50.8	53.0	50.3	51.2	51.3	2.87	0.57	0.85	0.48
NDF digestibility	45.4	49.1	51.9	46.7	48.7	48.3	3.54	0.79	0.80	0.36
ADF digestibility	40.5	43.0	43.3	38.2	38.9	38.0	4.40	0.72	0.74	0.66

Item:* expressed as g. 100 g DM^{-1}.

Water soluble carbohydrates: Low= 21 g.100 g DM^{-1} , Medium: 24 g. 100 g DM^{-1} , High 27 g. 100 g DM^{-1} ; SP (soluble protein) content: SPLow: 5.2 g.100g $DM^{-1,}$ SPHigh: 9.4 g.100g DM^{-1}. DM= dry matter, NDF= neutral detergent fiber, ADF: acid detergent fiber. WSC= water soluble carbohydrate main effect, SP = soluble protein main effect, WSC x SP = water soluble carbohydrate x soluble protein interaction.

Table 5.4. Nitrogen digestion and bacterial crude protein synthesis of diets differing in water soluble carbohydrates and soluble protein content, fed to continuous culture fermenters.

	Water soluble carbohydrates									
	LWSC		MWSC		HWSC		SEM	*p- value*		
SP content	LSP	HSP	LSP	HSP	LSP	HSP		WSC	SP	WSC x SP
*Item**										
Total N intake (g/d)	2.34	3.04	2.44	2.93	2.36	3.11				
Total N outflow ($g.d^{-1}$)	0.71	0.75	0.73	0.75	0.72	0.79	0.052	0.83	0.27	0.84
N digestion (%)	69.79	75.49	70.43	74.38	69.56	74.47	2.032	0.94	0.009	0.89
Bacterial CP synth (g/d)	1.76	1.51	1.88	1.64	1.82	2.19	0.062	<0.001	0.39	<0.001
Bact N / total N outflow	0.42	0.38	0.37	0.40	0.39	0.44	0.045	0.67	0.66	0.43
Bact CP ($g.100\ digDM^{-1}$)	8.87	8.57	8.06	9.00	8.63	10.50	1.190	0.58	0.34	0.61

Item:* expressed as g. 100 g DM^{-1}.

Water soluble carbohydrates: Low= 21 g.100 g DM^{-1} , Medium: 24 g. 100 g DM^{-1} , High 27 g. 100 g DM^{-1} ; SP (soluble protein) content: SPLow: 5.2 g.100g $DM^{-1,}$ SPHigh: 9.4 g.100g DM^{-1}. N= nitrogen. Bact CP = bacterial crude protein synthesis, expressed as g . d^{-1} and as g . 100 g of digested DM^{-1}.

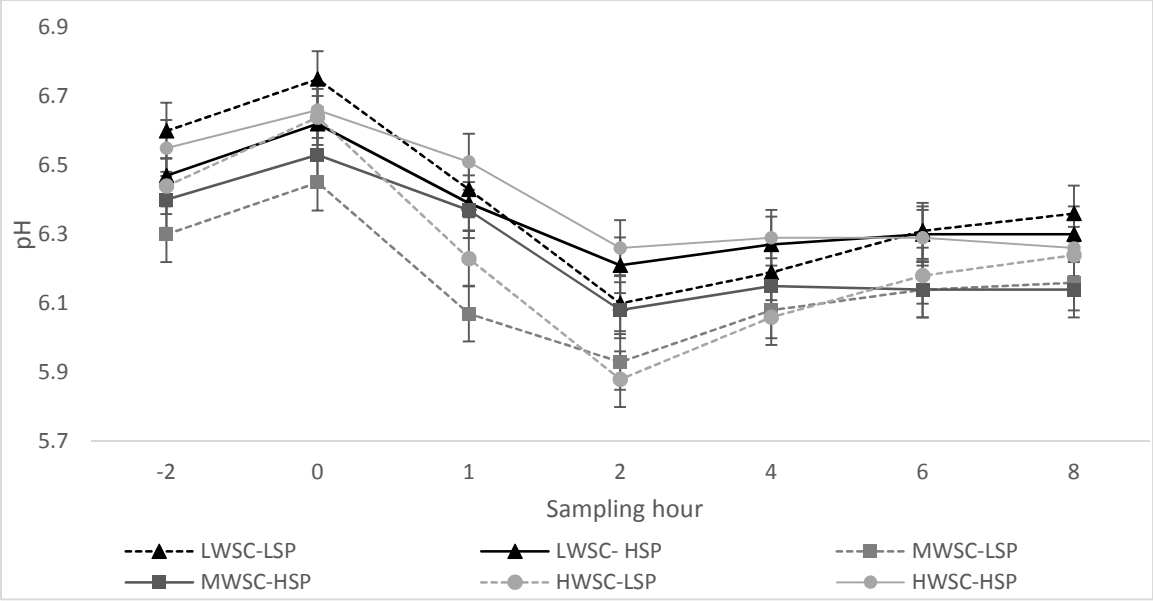

Figure 5.1. Daily continuous culture fermenters pH at three levels of water soluble carbohydrates content (LWSC, MWSC and HWSC, low, medium and high, respectively) and two levels of soluble protein content in diet (LSP and HSP, low and high, respectively). Error bars = 0.0810, standard error of the mean. Fermenters pH was not affected by WSC, SP or the WSCxSP interaction ($P > 0.05$). There was a sampling hour effect ($P < 0.0001$) and significant interaction between sampling hour and SP level ($P < 0.003$)

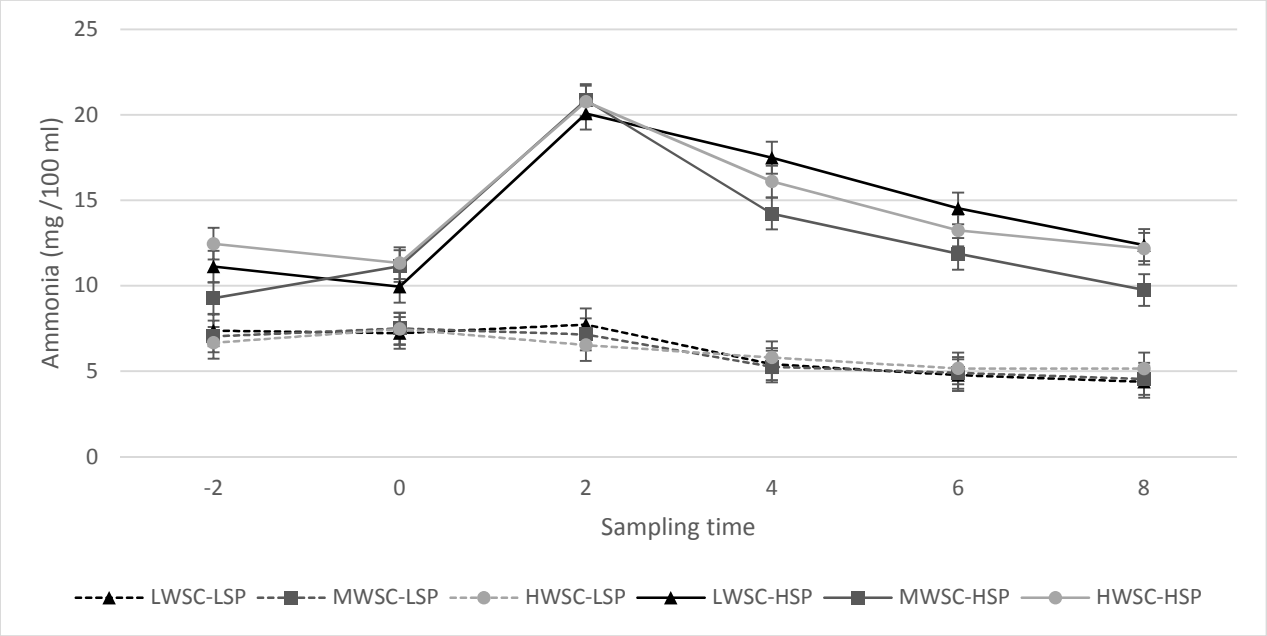

Figure 5.2. Daily continuous culture fermenters ammonia concentration at three levels of water soluble carbohydrates content (LWSC, MWSC and HWSC, low, medium and high, respectively) and two levels of soluble protein content in diet (LSP and HSP, low and high). Error bars = 0.928, standard error of the mean. Ammonia level was affected by SP level ($P < 0.0001$) but not by WSC level ($P > 0.05$). It was also affected by sampling hour ($P < 0.0001$) and there was a significant interaction between SP and sampling hour ($P < 0.0001$).

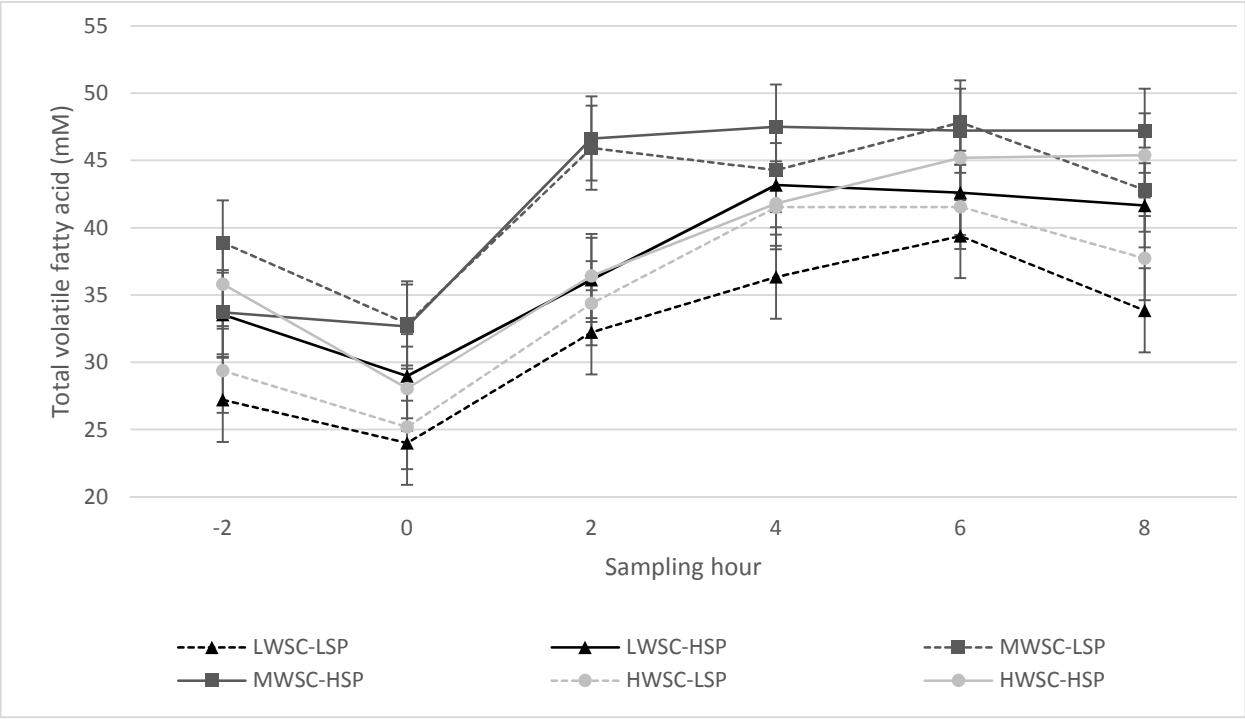

Figure 5.3. Daily continuous culture fermenters total volatile fatty acids concentration at three levels of water soluble carbohydrates content (LWSC, MWSC and HWSC, low, medium and high, respectively) and two levels of soluble protein content in diet (LSP and HSP, low and high). Error bars = 3.129, standard error of the mean. Total VFA was affected by sampling hour ($P < 0.0001$) and there was a significant interaction between sampling hour and WSC level ($P < 0.02$).

CHAPTER SIX

RESIDUAL FEED INTAKE IN CATTLE: PHYSIOLOGICAL BASIS. A REVIEW

ABSTRACT

Residual feed intake (RFI) is an estimate of feed efficiency independent of the level of production. Briefly, RFI is estimated as the difference between the observed and the expected intake for a given liveweight gain and metabolic body weight. Therefore, cattle with lower RFI are considered to be more efficient. No single biological mechanism explains the variability in RFI. Research has found that low RFI cattle generate less methane per unit of liveweight gain, but it is not clear if they yield less methane per unit of dry matter intake. Differences in digestion and rumen function have been reported. According to some evidence, low RFI cattle would have lower maintenance requirements, but results are inconclusive. Some evidence suggests they have a lower protein turnover. Activity and feeding behavior differ in cattle contrasting in RFI, and more efficient cattle would be less active and would show lower daily number of feeding events. Gain composition seems also related to RFI but it does not appear to be the main factor. Visceral weight, mitochondrial function and hormones have also been studied, with inconclusive results. Residual feed intake relies on multiple physiological traits and further elucidation will be important to implement successful selection programs in cattle.

INTRODUCTION

Even though feed costs are the main determining factor of beef industry profitability, genetic selection programs have historically been focused on increasing the individual animal output [i.e., liveweight gain] rather than on optimizing the inputs [i.e., feed intake] (Sainz and Paulino, 2004, Herd and Arthur, 2009). Traits such as gain to feed ratio (G:F) are used to summarize efficiency, but the final result may have been misleading. Gain to feed ratio is highly related to liveweight gain and does not necessarily improves the energy efficiency use, even though it can result in a dilution of the maintenance cost. Selecting on G:F can lead to selection for growth potential, which also leads to selection for mature size and higher dry matter (DM) intake (Barlow, 1984, Owens et al., 1995; Arthur et al., 2001). This method of selection might also have led to cattle which are very efficient when allowed *ad libitum* intakes (i.e., feedlot), but perform poorly when DM intake is limited, as usually occurs in grazing situations (Jenkins and Ferrell, 1994).

Residual Feed intake (RFI) is an indicator of feed efficiency independent of the level of production (Herd and Arthur, 2009). Originally, Koch et al. (1963) proposed that feed intake could be adjusted for body weight and weight gain and partitioned into two components: 1) intake expected for a given performance or level of production, and 2) individual deviation from the expected value based on the regression line (i.e., residual portion). As a result of this calculation, animals that showed DM intake lower than the expected for a given weight gain would have lower (negative) RFI and would be more efficient. The progeny would also show the feature (Arthur et al., 2001, Herd et al., 2004).

In practical terms, RFI is calculated as the difference between actual DM intake and expected DM intake, with data obtained from the individual measurements of daily feed intake and average daily gains in long term feeding trials (at least 70 to 84 days, Sainz and Paulino, 2004). Expected DM intake (eDMI) is in turn calculated using a multiple regression model, using metabolic body weight (MBW) and average daily gain (ADG) as independent variables, as follows:

$$eDMI_j = \beta_0 + \beta_1 MBW + \beta_2 ADG + e_j$$

Since the variability in RFI was acknowledged, there has been abundant research to assess the physiological mechanisms underlying it (Herd et al., 2004, Herd and Arthur, 2009). Evidence shows that no single biological mechanism is responsible for the observed variability (Herd et al., 2004). At least theoretically, every physiological step that affects the conversion of gross energy contained in feed to animal product (i.e., meat) can be considered potentially responsible for the observed variability in RFI. Therefore, understanding these factors is an important prerequisite before implementing an effective breeding strategy towards more efficient animals (Nkrumah et al., 2006). Research indicates that RFI has a moderate heritability ($h^2 = 0.3 – 0.4$, Herd and Bishop, 2000, Arthur et al., 2001). However, given its complex nature and the underlying multiple biological processes, genetic selection on the base of RFI has not always been successful (Karisa et al., 2014). The present review focuses on the factors that affect the RFI in cattle.

RESIDUAL FEED INTAKE AND ITS RELATION WITH ANIMAL PERFORMANCE

Predictably, RFI is positively correlated with feed intake (r = 0.66; Rolfe et al., 2011); however, numerous reports show that genetic correlations between RFI and ADG or MBW are close to zero (Arthur et al., 2001, Richardson et al., 2004, Castro Bulle et al., 2007, Rolfe et al., 2011, Fitzsimons et al., 2013, Perkins et al., 2014). Rolfe et al. (2011) reported a strong and negative genetic correlation between G:F and RFI (r = 0.92). Arthur et al. (2001) found that a selection based on G:F leads to selection for greater ADG, which in turn leads to larger mature size. On the contrary, a selection plan based on RFI should not lead to an increase in animal mature size, yielding more efficient cattle, able to perform adequately when feed resources are scarce.

GENETIC BASIS OF RFI

Since Koch et al. (1963) first introduced the concept of using residuals to express production efficiency, numerous authors have focused on dissecting the genetic components of RFI. RFI has been found to be moderately heritable by multiple authors (e.g., Arthur et al., 2001, Rolfe et al., 2011, Saatchi et al., 2014) and as a consequence should respond favorably to selection. The availability of high-density genomic information via tens to hundreds of thousands of single nucleotide polymorphisms (SNP) has allowed for the dissection of the heritable fraction of RFI at the genomic level. In example, Saatchi et al. (2014) discovered ten significant 1-Megabase SNP windows located on eight autosomes for RFI with the largest effect 1-Megabase SNP window detected on

chromosome 15 in a Simmental x Angus population which explained 2.40% of the additive genetic variance. Large-effect QTL associated with RFI were also detected on chromosomes 6, 10, 14, 18, 19, 20 and 25.

BIOLOGICAL BASIS FOR THE VARIABILITY IN RFI

Methane emission

Methane (CH_4) production is the most important "hydrogen sink" in the process for regenerating oxidized co-factors (NAD^+) in the rumen. Methane production per mole of fermented hexose is higher in high fiber diets than in high grain diets, due to differences in rumen metabolic pathways (Fahey and Berger, 1988). Acetate and butyrate production promotes more CH_4 emission, while propionate is considered a competitive pathway for hydrogen uptake in the rumen (Moss et al., 2000). Methane production is an energy loss from the rumen, affecting the overall energy use efficiency and represents between 2 to 12% of the total gross energy intake (Nagaraja, 2012). In the last few years, great attention has been paid to the emission of CH_4 from ruminants due to its contribution to global warming (Moss et al., 2000).

Selecting for lower RFI could yield cattle that produce less CH_4 while attaining the same performance, thus reducing the environmental impact of beef and milk production. However, the basis on which these differences rely remains unclear. Fitzsimons et al. (2013) reported lower methane production in low RFI cattle, both when expressed in g $CH_4.d^{-1}$ or g CH_4. g MBW^{-1}. However, no differences were detected when methane

production was expressed in g CH_4. kg DMI^{-1}. Hegarty et al. (2007) also reported a positive correlation between RFI and CH_4 production. They compared steers with high and low RFI and found that the steers with low RFI produced 24% less methane per unit of ADG. However, similar to Fitzsimons et al. (2013) findings, no differences in CH_4 yield per kg of DM intake were found. Similar findings were reported by Waghorn and Hegarty (2011). Together, these results suggest that the mechanism underlying the lower CH_4 production by low RFI cattle would depend on the lower total DM intake rather than on differences in rumen metabolism (Fitzsimons et al., 2013). However, Nkrumah et al. (2006) found that cattle selected for lower RFI produced less CH_4 per kg of MBW or per kg of ADG and that these differences persisted even when DM intake was used as a covariate, indicating that mechanisms other than lower DM intakes might be underlying the lower CH_4 production.

Recently, Carberry et al. (2014) reported no differences in the total abundance of methanogens between cattle contrasting in RFI. These results agree partially with those of Zhou et al. (2009), who also found no differences in the total population of methanogens, but reported differences in the composition, diversity and proportion of methanogen species between high RFI and low RFI. However, none of the studies attempted to relate the differences in methanogen population with total CH_4 production.

Regardless if differences are due to ruminal microbial composition or simply to a lower DMI, it is clear that low RFI cattle produce less CH_4 per unit of production (i.e., kg of gain). Each 1 $kg.d^{-1}$ increase in RFI is associated with between 14 $g.d^{-1}$ (Hegarty et al., 2007) and 26 $g.d^{-1}$ (Fitzsimons et al., 2013) of additional CH_4 emission. However, most studies have been done with cattle consuming high concentrate diets. The mitigating effect

of low RFI on CH_4 production might be dependent on diet quality. For example, Jones et al (2011) found no differences in CH_4 production between heifers selected divergently on RFI when they grazed a low quality pasture (55% digestibility). However, when the same set of animals grazed a high quality pasture (82% digestibility), those selected by lower RFI had 27% lower CH_4 emission. Additionally, the lower CH_4 production was accompanied by a reduced DM intake.

Digestibility and rumen function

Nkrumah et al (2006) found a tendency for a higher DM and crude protein digestibility in cattle selected for lower RFI. Similar results were reported by Richardson et al. (1996), who estimated that differences in digestibility would explain 19% of the total variation in RFI. It has been suggested that differences in DM intake, ruminal retention time and feeding behavior could be the mechanism underlying the higher digestion efficiency in low RFI cattle (Nkrumah et al., 2006). However, several other authors have found no relation between digestibility and RFI (Cruz et al., 2010, Gomes et al., 2013, Fitzsimons et al., 2013, 2014). Herd and Arthur (2009) advised caution in allocating digestibility as a major factor determining RFI, mainly because of the difficulties in accurately detecting differences in digestibility.

Evidence of differences in rumen metabolism between cattle differing in RFI exists. However, results are contradictory and evidence is far from conclusive (Lawrence et al., 2011, 2013, Fitzsimons et al., 2013, 2014). Fitzsimons et al. (2014) reported no differences in rumen pH, VFA proportions or lactic acid concentration. However, a previous report

from the same group of researchers (Fitzsimons et al., 2013) found that cattle with lower RFI tended to have a higher propionate concentration and a lower acetate:propionate ratio in rumen, which agrees with results reported by Lawrence et al. (2011, 2013) but disagrees with the results of Kruegger et al. (2009), who found exactly the opposite. Diet composition and fiber content might probably interact with RFI, explaining partially this disagreement. With respect to microbial diversity, McCann et al. (2014) analyzed the rumen diversity and species of grazing Brahman cattle differing in RFI and found only slight differences in bacterial population. Cattle differing in RFI occasionally show differences in digestion and rumen function, but results are inconclusive and disagreement exists on the influence of this in determining differences between animals for RFI.

Metabolism, maintenance and heat production

Some research suggests that low RFI cattle could have lower maintenance requirements (Archer et al., 1999, Herd and Bishop, 2000, Castro Bulle et al., 2007, Gomes et al., 2012), although evidence is not totally conclusive. Since an accurate and "true" measure of maintenance requirements implies long term experiments, most of the estimations are obtained indirectly. For example, Herd and Bishop (2000) estimated maintenance as the difference between the total ME intake and the ME used for growth, taking into account the gain composition and using standard efficiencies for fat and protein deposition. Nkrumah et al. (2006) assessed heat production indirectly using oxygen consumption and found that heat production was decreased 21% in low RFI *versus* high RFI steers. Consistently, the energy retention was higher. The authors concluded that these

differences were independent of the level of DM intake. Basarab et al. (2003) calculated heat production from ME intake and gain composition and estimated that low RFI cattle produced leas heat. Montanholi et al. (2010) used infrared thermography, which measures the body surface temperature, to assess the heat production of cattle. The authors found that low RFI cattle showed lower surface temperature in the eye, cheek and snout regions, and concluded that this was an indicator of lower heat production in more efficient steers. However, this results should be taken carefully, since heat production is affected by DM intake, and estimations would be more accurate if done under similar DM intakes.

Protein turnover is an energy demanding process which accounts for an important portion of the total basal metabolic rate (Richardson et al., 2004) and strongly influences maintenance requirements. Castro Bulle et al. (2007) estimated that the maintenance requirements increased by 16.6 kcal. kg MBW. d^{-1} for each percentage increase in protein breakdown. Therefore, several researchers have looked for relations between RFI and protein turnover rate. Richardson et al. (2004) reported that less efficient cattle (higher RFI) showed higher levels of total plasma protein, urea and aspartate aminotransferase, all possible indicators of increased protein catabolism. However, the authors did not find differences in urine 3-methyl histidine:creatinine ratio, which is an indicator of rate of muscle breakdown. Castro Bulle et al. (2007), working with *Bos taurus,* as well as Gomes et al. (2013) working with *Bos indicus,* compared high and low RFI steers, finding no differences in 3-methyl histidine:creatinine ratio nor in estimated fractional protein degradation, synthesis or accretion rate. However, McDonagh et al. (2001) reported higher calpastatin activity (+13%) with no differences in calpain activity in low RFI cattle. Since

the calpain-calpastatin system is related to the rate of protein breakdown in the live animal, with calpastatin being an inhibitor of protein breakdown, these results would indicate that low RFI steers could have a lower protein turnover, reducing the metabolic impact of this energy demanding process.

Other processes occurring at cell level have been proposed as partially responsible for variations in feed efficiency (Herd and Arthur 2009, Karisa et al., 2014). Great attention has been paid to mitochondrial proton leak and ion pumping associated with Na+/K+ ATPase (Cartens and Kerley, 2009). Mitochondria possess an efficient mechanism that allows capturing the energy generated by the electronic transport, and use it to pump protons against a gradient into the intermembrane space and then take advantage of that gradient by coupling the proton flux with the synthesis of ATP. However, sometimes this highly efficient mechanism can be uncoupled, and the protons leak back into the mitochondrial matrix generating heat rather than ATP, in a process that dissipates energy (Stuart et al., 1999, Harper et al., 2002, Neufer, 2015). This waste of energy is thought to account for at least 20% of the basal metabolism of rats (Nobes et al., 1990, Rolfe and Brand, 1996). Proton leak can occur both by simple diffusion through the lipid bilayer of the inner mitochondrial as well as facilitated by proteins, known as uncoupling proteins, of which many isoforms have been described (Stuart et al., 1999). The importance of mitochondrial proton leak in energy efficiency has been noted by researchers, who aimed to elucidate its importance in livestock. Both in poultry (Ojano-Dirain et al., 2007, Bottje and Cartens, 2009) and pigs (Grubbs et al., 2013, Lonergan, 2015), it has been shown that proton leakage is related to RFI. In cattle, Kolath et al. (2006) compared mitochondrial

function in low and high RFI and did not find differences in proton leakage, however more efficient cattle surprisingly had an increased rate of respiration. Simielli-Fonseca et al. (2015) analyzed the expression of mitochondrial proteins in Nelore cattle and found that low RFI (more efficient) showed higher expression of certain isoforms of uncoupling proteins in the liver and no difference in uncoupling protein expression was found in the muscle. Similarly, no difference in proton leak kinetics in beef cattle hepatocytes were reported by Lancaster et al. (2014). Therefore, evidence of mitochondrial function differences in cattle is far from conclusive. Further research is needed in this area.

Visceral weight

Given their high metabolic activity per unit of tissue, visceral organs are responsible for a large proportion of oxygen consumption and heat production (Reynolds, 2002). The gastrointestinal tract and liver, representing 7.0 and 2.5 % of body weight, respectively, account for more than 40% of the total energy demands of the body (McBride and Kelly, 1990). Therefore, there is a negative correlation between energy efficiency and visceral organ size, which in turn is also affected by total DM intake (Johnson et al., 1990). The abovementioned explains why there has been an interest in correlating visceral weight and RFI. Basarab et al. (2003) reported that high RFI steers had heavier liver, stomach and intestine than low RFI steers. Fitzsimons et al. (2014) found a higher ruminal reticular weight in high RFI bulls compared to low RFI bulls, but did not detect differences in weight for the rest of the splanchnic organs, including liver and intestine. On the other hand, other

authors did not find any relationship between total visceral organ mass and RFI (Richardson et al., 2001, Cruz et al., 2010).

Feeding behavior and activity

In a review, Herd and Arthur (2009) assigned 10% of variability in cattle RFI to physical activity and 2% to feeding patterns. Richardson et al. (1999) reported a positive phenotypic correlation (r=0.32) between RFI and total steps measured by a pedometer, suggesting that activity is an important factor influencing RFI. There is also evidence that more efficient cattle spend more time lying down (Gomes et al., 2013). Additionally, feeding behavior traits, like frequency and duration of feeding events, showed significant differences between cattle differing in RFI (Gomes et al., 2013). Robinson and Oddy (2004) recognized three feeding behavior traits associated with RFI. Higher RFI was phenotypically positively correlated with longer daily eating time ($min.d^{-1}$), more events of bunk attendance and faster rate of eating ($g\ DM.min^{-1}$). Coincidently, Nkrumah et al., (2006) recorded the feeding duration ($min.d^{-1}$) and the number of events of bunk attendance and found that animals selected for lower RFI spent 36% less time eating and had a reduced number of events of bunk attendance. Similar results are reported by Golden et al. (2008), Montanholi et al., 2010 and Gomes et al. (2013). Additionally, data shows that higher RFI cattle have a more variable temporal pattern of feed intake (Golden et al., 2008, Dobos and Herd, 2008). According to the evidence, it seems that feeding behavior and eating patterns, as well as physical activity, differ in cattle divergently selected for RFI. However,

according to Golden et al. (2008) these correlations, although significant, are not strong enough to allow for an accurate prediction of RFI from feeding behavior assessment alone.

Body composition and meat quality

Per unit of weight, the deposition of lean tissue is less energetically costly than fat. Gross energy of fat and protein are 9.38 $Mcal.kg^{-1}$ and 5.54 $Mcal.kg^{-1}$, respectively (Garrett and Hinman, 1969). Additionally, muscle contains a water to protein ratio in average of 4:1, whereas fatty tissue contains very low water concentration (Gerrard and Grant, 2003). Therefore, variations in gain composition affect the nutrient efficiency use (Herd and Arthur, 2009).

Richardson et al. (2001) analyzed the progeny of cattle divergently selected for RFI. The authors suggested that carcass chemical composition was correlated to RFI, with progeny of low RFI (more efficient) cattle having less carcass fat and more protein. However, less than 5% of the total variation in RFI was explained by variation in body composition. Basarab et al. (2003) found high RFI cattle had slightly higher empty body fat gain, and that marbling score and backfat thickness were positively correlated to RFI. The authors estimated that 6.8% of the variation in RFI was explained by variation in gain of empty body fat. Similarly, slight differences or weak positive correlations between RFI and subcutaneous fat thickness have been reported by several other authors (Herd and Bishop, 2000, Mc Donagh et al., 2001, Arthur et al., 2001, Schenkel et al., 2004). On the other hand, no ultrasound differences for back and rump fat thickness were detected by

Fitzsimons et al (2014) between high and low RFI bulls. Additionally, the authors detected no significant differences in carcass composition except for a moderate correlation with dressing percentage. In fact, numerous studies have found no correlation between RFI and fat proportion and fat:lean ratio (Mader et al., 2009, Cruz et al., 2010, Fitzsimons et al., 2014, Perkins et al., 2014). Similarly, percentage of subcutaneous fat and body cavity fat has not been correlated with RFI (Basarab et al., 2003)

From a selection standpoint, any potential improvement in cattle feed efficiency might be worthless if obtained at the expense of meat quality (Fitzsimons et al., 2014). Some of the evidence reviewed gives reason for two possible concerns derived from ongoing selection, which are potential reductions in both marbling score and tenderness. Marbling score, a visual indicator of the intramuscular fat content, is a factor affecting beef palatability and, in some countries, higher marbling scores result in premium prices. Therefore, establishing the impact of selection based on RFI on marbling and IM fat content has been a concern for many researchers. Nkrumah et al. (2007) reported moderate positive genetic correlations between RFI and marbling assessed either by ultrasound (r=0.32) or measured in the carcass postmortem (r=0.28). Similar correlations were reported by Ahola et al. (2011). However, other authors have reported no correlation between RFI and IM fat assessed by ultrasound (Schenke et al., 2004, Shaffer et al., 2011) or marbling score assessed postmortem (McDonagh et al., 2001, Perkins et al., 2014).

Evidence indicates that RFI could be linked to calpastatin expression and lower myofibril fragmentation index in low RFI cattle (Mc Donagh et al., 2001). Therefore, potential impacts of selection on beef sensorial quality, mainly tenderness, have been

assessed. Ahola et al. (2011) found no effect of divergent RFI selection on beef sensory traits assessed by a trained panel (tenderness, juiciness, flavor and presence of off-flavors). They also did not find differences in Warner-Bratzler shear force. Similarly, Mc Donagh et al. (2001) reported no differences in Warner-Bratzler shear force or compression values nor in muscle and fat color in steers differing in RFI.

The correlation between body compositions and RFI, although significant in some studies, is low and does not appear to be a determining factor of differences in efficiency. However, to reduce the risk that selection against RFI could lead to leaner cattle, some authors (Schenkel et al., 2004) have suggested to include an adjustment using backfat thickness as an extra coefficient in the regression. Although great effects on meat quality have not been detected, continuous selection may theoretically lead to changes in tenderness. Since most of the reported experiment are a single generation of divergent selection, potential impacts of prolonged selection for RFI may be clearer when multi-generational information is attained.

Hormones

Associations between hormone concentrations and RFI have been studied. Leptin is a peptidic hormone expressed mainly by white adipose tissue (Ahima and Flier, 2000) which has been considered not only as a sensor of adiposity level but also as a regulator of energy consumption (Houseknecht et al., 1998) and a hastener of oxygen consumption through increased metabolic rate (Scarpace et al., 1997, Chilliard et al., 2005) . Results are

inconclusive with respect to the correlation between leptin levels and RFI. Richardson et al. (2004) reported higher leptin concentration in high RFI steers, which the authors associated with a higher fat mass (Frederich et al., 1995, Chilliard et al., 2005). On the other hand, Perkins et al., (2014) found leptin mRNA expression in adipose tissue to be 245% higher in low RFI than in high RFI steers, which the authors found consistent with the lower intakes observed in the low RFI steers.

Perkins et al. (2014) also studied gene expression of a series of hypothalamic neuropeptides controlling feed intake in high and low RFI steers. Low RFI showed a higher expression of genes linked to the synthesis of anorexigenic peptides (Pro-opiomelanocortin, precursor of α-MSH) and a lower expression of Neuropeptide-Y and relaxin-3, which stimulate consumption (orexigenic). The authors suggested that this could be the base of the differences in feeding behavior and DMI in cattle differing in RFI. Additionally, RFI showed a relation with the expression of hypothalamic and pituitary hormones linked with the reproductive physiology, but the relation between RFI and reproductive axis remains unclear.

Moore et al. (2005) has shown that RFI is positively related with plasma IGF-1 levels, which in turn is correlated positively with subcutaneous and intramuscular fat content. The authors suggested that selecting for lower IGF-1 levels could lead to more efficient and leaner cattle. Several authors have also reported an association between the GH and RFI (Karisa et al., 2013, Kelly et al., 2013), although others have found no correlation (Lancaster et al., 2008).

Research (Richardson et al., 2004, Gomes et al., 2013) has also reported a higher cortisol plasma concentration in high RFI steers, leading them to conclude that high RFI could be more susceptible to stress and this could be part of the explanation for differences in efficiency.

CONCLUSION

No single mechanism is responsible for the differences in RFI and nutrient use efficiency. RFI can be considered a restricted selection index, containing multiple components. A better understanding of the underlying physiological process will help to improve selection programs.

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