Effects of ensiling conditions on the nutritional quality of forage legumes and their impacts on rumen fermentation and nutrient utilization by cattle

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Summary

Silage produced from forage legumes can contribute to the limiting protein supply of ruminants' diets in the tropics, and reduced dependence on imported and high-carbon footprint feeds. However, the successes recorded with temperate forage legume silage feeding in ruminants have not been achieved in the tropics. Thus, the effects of silage feeding on ruminants' performance cannot be isolated from the processes that occur during ensiling. Since controllable and uncontrollable factors govern silage quality, it is imperative to understand the processes that occur during ensiling tropical forage legumes under different conditions to widen knowledge. Therefore, the aim was to determine the effect of ensiling conditions on forage legume nutritional quality, their ruminal and post-ruminal fate, and their potential impact on nutrient utilization by cattle in the tropics.

A silage study was conducted to evaluate the effects of ensiling length and storage temperature on the nutritive value and fibre-bound protein of three tropical forage legumes ensiled alone or combined with sorghum. The three forage legumes included soybean (*Glycine max*), lablab (*Lablab purpureus*) and jack bean (*Cannavalia ensiformis*). Silages from each legume were made individually or combined with sorghum (*Sorghum bicolor*) and stored outdoors or indoors for 30, 75, and 180 days. The results showed that the proportion of soluble nutrients preserved in most silage until 75 d of ensiling declined considerably, thereby increasing dry matter (DM) and crude protein (CP) losses, fibre concentration and reducing digestibility afterwards. Besides, storage temperature affected the fermentation and fibre-bound protein characteristics with higher variation in legume silages' fibre-bound protein than the sorghum-legume silages.

Silages of sorghum and soybean were selected from the first study to compose low and high CP diets with additional ingredients, and the effects of ensiling length, storage temperature, and its interaction with CP levels on in vitro rumen fermentation and post-ruminal digestibility were assessed. Dietary treatments were incubated in duplicate for 8 and 24 h in three runs using the ANKOM RF technique to study rumen fermentation. Post-ruminal digestibility was determined using the pepsin and pancreatic solubility procedure. The results showed that gas production (GP) and ammonia-nitrogen in the rumen inoculum increased quadratically with the ensiling length, with the highest GP and ammonia-nitrogen at 75 d of ensiling, irrespective of incubation times. The GP was higher in diets with low than high CP concentrations, while it was the opposite for ammonia-nitrogen. An interaction between ensiling length and storage temperature effect was found for the apparent CP intestinal digestibility. Overall, ensiling beyond 75 d reduces CP digestibility to the extent that it cannot be recovered by supplying additional CP.

In the third study, the effects of CP levels on nutrient intake, digestibility, nitrogen metabolism and performance of growing steers fed corn or corn-soybean silage were investigated. Sixteen growing steers were fed with rations based on corn or corn-soybean silage at high or low CP levels in a 4 × 3 incomplete Latin square design comprising 17 d periods, each with 12 d of adaptation to dietary treatments and 5 d of sampling. While the effect of silages and CP levels were not found for nutrient intake, the apparent total tract digestibility of nutrients was reduced for low than high CP in both silages, with greater differences between the CP levels in corn than corn-soybean silage. The average daily gain and feed efficiency were greater in low than high CP of corn silage, but no differences between CP levels were found in corn-soybean silage. In general, corn silage with low CP concentration but with a high

metabolizable energy supply supposedly improved nitrogen use efficiency with a higher yield of microbial protein and average daily gain than other diets.

Conclusively, the results of the current thesis showed that ensiling forage legumes individually or in combination with cereal crops beyond 75 d at high temperatures of the tropics leads to a decline in the nutritional quality of legume silage and CP intestinal digestibility even with additional CP sources. Furthermore, prolonged ensiling of combined legume and cereal crops reduces nutrient availability for cattle performance.

Zusammenfassung

Silage aus Futterleguminosen kann dazu beitragen, die eingeschränkte Eiweißversorgung von Wiederkäuern in den Tropen zu verbessern und die Abhängigkeit von importierten Futtermitteln mit hohem Kohlenstoffausstoß zu verringern. Die Erfolge, die bei der Silagefütterung von Wiederkäuern mit Futterleguminosen in den gemäßigten Breiten erzielt wurden, wurden in den Tropen jedoch nicht erreicht. Daher können die Auswirkungen der Silagefütterung auf die Leistung der Wiederkäuer nicht von den Prozessen isoliert werden, die während der Silierung ablaufen. Da kontrollierbare und unkontrollierbare Faktoren die Silagequalität beeinflussen, ist es unerlässlich, die Prozesse zu verstehen, die während der Silierung tropischer Futterleguminosen unter verschiedenen Bedingungen ablaufen, um unser Wissen zu erweitern. Ziel war es daher, die Auswirkungen der Silierbedingungen auf die Nährstoffqualität von Futterleguminosen zu ermitteln, was mit ihnen im Pansen und danach passiert sowie ihre möglichen Auswirkungen auf die Nährstoffverwertung durch Rinder in den Tropen.

In einer Silierstudie wurden die Auswirkungen der Silierdauer und der Lagertemperatur auf den Nährwert und das fasergebundene Protein von drei tropischen Futterleguminosen untersucht, die allein oder in Kombination mit Sorghum siliert wurden. Bei den drei Futterleguminosen handelt es sich um Sojabohne (*Glycine max*), Helmbohne (*Lablab purpureus*) und Jackbohne (*Cannavalia ensiformis*). Es wurden Silagen der einzelnen Leguminosen und in Kombination mit Sorghum (*Sorghum bicolor*) hergestellt und 30, 75 und 180 Tage lang im Freien oder in einem Raum gelagert. Die Ergebnisse zeigten, dass der Anteil der löslichen Nährstoffe in den meisten Silagen bis 75 Tage nach der Silierung erhalten blieb, danach jedoch beträchtlich abnahm, wodurch die Verluste an Trockensubstanz (TS) und Rohprotein (XP) sowie die Faserkonzentration zunahmen und die Verdaulichkeit verringert wurde. Außerdem wirkte sich die Lagertemperatur auf die Fermentation und die Eigenschaften der fasergebundenen Proteine aus, wobei das fasergebundene Protein der Leguminosen-Silagen stärker variierte als das der Sorghum-Leguminosen-Silagen.

Silagen aus Sorghum und Sojabohnen wurden aus der ersten Studie ausgewählt, um Futtermittel mit niedrigem und hohem XP-Gehalt und zusätzlichen Bestandteilen zusammenzustellen, und die Auswirkungen der Silierdauer, der Lagertemperatur und ihrer Wechselwirkung mit dem XP-Gehalt auf die In-vitro-Pansenfermentation und die postruminale Verdaulichkeit wurden bewertet. Die Futterbehandlungen wurden in zweifacher Ausführung in drei Wiederholungen für 8 und 24 Stunden mit der ANKOM RF-Technik zur Untersuchung der Pansenfermentation inkubiert. Die postruminale Verdaulichkeit wurde mit Hilfe des Pepsin- und Pankreaslöslichkeitsverfahrens bestimmt. Die Ergebnisse zeigten, dass die Gasproduktion (GP) und der Ammoniakstickstoff Konzentration im Panseninokulum quadratisch mit der Silierdauer zunahmen, mit der

höchste GP und dem höchsten Ammoniakstickstoff Konzentration bei 75 Tagen Silierdauer unabhängig von der Inkubationszeit. Die GP war bei Futtermitteln mit niedrigem XP-Gehalt höher als mit hohem XP-Gehalt, während es sich beim Ammoniakstickstoff umgekehrt verhielt. Für die scheinbare XP-Verdaulichkeit im Darm wurde eine Wechselwirkung zwischen der Silierdauer und dem Effekt der Lagertemperatur festgestellt. Insgesamt verringert die Silierung nach 75 Tagen die CP-Verdaulichkeit in einem Maße, das nicht durch zusätzliche CP-Zufuhr ausgeglichen werden kann.

In der dritten Studie wurden die Auswirkungen des XP-Gehalts auf die Nährstoffaufnahme, die Verdaulichkeit, den Stickstoffmetabolismus und die Leistung von heranwachsenden Ochsen untersucht, die mit Mais- oder Mais-Sojabohnen-Silage gefüttert wurden, untersucht. Sechzehn heranwachsende Ochsen wurden 17 Tage mit Rationen auf der Basis von Mais- oder Mais-Sojabohnen-Silage mit hohem oder niedrigem XP-Gehalt gefüttert (12 Tage zur Anpassung an die Ration und 5 Tage zur Probennahme), wobei ein 4 × 3 unvollständiges lateinisches Quadrat-Design verwendet wurde. Während bei der Nährstoffaufnahme keine Auswirkungen der unterscheidlichen Silagen und des XP-Gehalts festgestellt wurden, war die scheinbare Verdaulichkeit der Nährstoffe bei beiden Silagen mit niedrigem XP-Gehalt geringer als mit hohem XP-Gehalt, wobei die Unterschiede zwischen den XP-Gehalten bei der Mais-Silage größer war als bei der Mais-Sojabohnen-Silage. Die durchschnittliche Tageszunahme und die Futtereffizienz waren bei der Maissilage mit niedrigem XP höher als bei der Maissilage mit hohem XxP, während zwischen den verschiedenen XP-Gehalten der Mais-Sojabohnen-Silage keine Unterschiede festgestellt wurden. Generell gilt, dass Maissilagen mit niedrigem XP-Gehalt, aber hoher metabolisierbarer Energiezufuhr voraussichtlich zu einem höheren Ertrag an mikrobiellem Protein sowie einer höheren

durchschnittlichen Tageszunahme führen und dadurch im Vergleich zu anderen Futtermitteln die Stickstoffverwertung verbessern.

Zusammenfassend zeigen die Ergebnisse der vorliegenden Arbeit, dass die Silierung von Futterleguminosen einzeln oder in Kombination mit Getreidepflanzen über 75 Tage hinaus bei hohen Temperaturen in den Tropen zu einer Verschlechterung der Nährstoffqualität der Leguminosensilage und der intestinalen Verdaulichkeit von XP führt, selbst wenn zusätzliche XP-Quellen verwendet werden. Darüber hinaus verringert eine längere Silierung von Leguminosen in Kombination mit Getreide die Verfügbarkeit von Nährstoffen und dadurch die Leistungsfähigkeit der Kühe.

Chapter 1

1. General introduction

1.1 Background

It is foreseeable that the global demand for food will quadruple in the next three decades from now due to population growth rate, increased income, and urbanization (United Nations, 2015). Remarkably, the share of animal-derived products in the total food demand globally is anticipated to increase by 50% in the next 30 years due to consumer preference that is likely to be influenced by societal development, particularly in developing countries (GASL, 2019). Though the per capita meat consumption in developing countries would be 37 kg per capita by the year 2030 as predicted by the FAO statistics, this is still insignificant compared to the prediction of 150 kg per capita in developed countries (FAOSTAT, 2015). Undoubtedly, the rise in demand for animal products in developing countries will lead to the intensification of animal production, thereby exerting environmental pressure on the food system in the absence of technological change. The increasing use of intensification of ruminant production by fattening with grain-based feeds poses threats to food and nutrition security due to competition with humans for grains. Considering that ruminants are natural converters of forages and crop residues into high-value animal products, leveraging on these roles of ruminants remain vital to mitigate food and nutrition insecurity in the face of the increasing human population (Mlambo and Mnisi, 2019). Thus, it is imperative that the use of forage resources currently available are optimized to support a high level of productivity in ruminants to reduce dependency on cereal and legumes grains. Unfortunately, of these forage resources, forage legume adoption is relatively poor across all tropical farming systems (White et al., 2013).

1.2 Forage legumes

Forage legumes belong to the *Fabaceae* (*Leguminosae*) and are generally defined by their podded grain, stipulated leaves, uncommon flower morphology, and the potential of 88% of the species to nodulate with rhizobia (Graham and Vance, 2003). About 750 genera of the *Fabaceae* are quite diverse and universal, with an estimated 18,000 to 19,000 species of legumes which include grain, pasture, and non-pasture species (Polhill, 1981). The number of these estimated species used as forages globally is not known totally, but 153 different legume species have been listed as forage legumes from the tropics and subtropics by an online open-

access animal feed resources information system (www.feedipedia.org; Phelan et al., 2015). The importance of forage and grain legumes cannot be overemphasized because they account for 27% of primary crop production worldwide, with 33% of grain legumes contributing to human dietary protein needs (Vance et al., 2000).

Legumes hallmark trait is the potential to produce root nodules and to fix atmospheric nitrogen in symbiotic association with Rhizobium bacteria. This ability makes forage legumes thrive in nutrient-depleted environments, thereby playing a critical role in natural ecosystems, agriculture, and agroforestry (Graham and Vance, 2003). Unfortunately, the yields of forage legumes are unmatched with those of grasses despite the genetic improvement, and yields are even lower in the tropics and subtropics as a result of the environmental and edaphic conditions as well as the high prevalence of diseases and pests in these regions (Graham and Vance, 2003). Additionally, the incorporation of improved legumes into farming ecosystems of the tropics and subtropics has lingered due to high seed costs, poor infrastructural facilities, and lack of information (Graham and Vance, 2003).

Successful integration of forage legumes into livestock systems in the tropics and subtropics is complex because it requires the evaluation of legume types, planting time, and management practices (Muir et al., 2014). The favourable outcome recorded in the temperate zones with the use of forage legumes such as alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) in ruminant production has not been achieved in the tropics. The diverse forage legumes in the tropics with different growth habits, a diverse range of plant secondary metabolites and different environmental niches might have affected the identification of a particular legume species that can match the agronomic persistence and nutritional quality of temperate forage legumes (Phelan et al., 2015). Additionally, the typically high temperatures in the tropics and subtropics amplify the differences in the leaf and stem anatomy and morphological characteristics of temperate and tropical legumes by decreasing the soluble nutrient fraction of forage legumes, thereby increasing fibre concentration and reducing fibre digestibility (Norton and Poppi, 1995).

In general, legumes are classified as protein crops, as they tend to have higher protein concentrations than grasses and cereals. The average crude protein (CP) concentration of forage legumes across a broader range of species and regions was found to be 170 g/kg dry matter (DM) in comparison with 115 g/kg DM for grasses (Dewhurst et al., 2009; Groff and

Wu, 2005; Minson, 1990). Thus, the utilization of forage legumes in ruminant feeding has been suggested as a means to improve production and reduce dependence on imported and high-carbon footprint feeds in the Tropics (Peters and Lascano, 2003). However, the high CP concentration of forage legumes alone is insufficient to measure the protein value for ruminants because, substantial CP proportions of forage legumes that are degraded in the rumen may not contribute to microbial CP synthesis and may not be hydrolyzed in the intestine (Broderick, 1995). Forage legumes are fed to ruminants in different forms (i.e., fresh, hay, silage, haulms), and the form of feeding have been shown as a contributing factor to the variability in the effects of legume feeding due to nutrient losses during harvesting and processing for a conservation purpose (Castro-Montoya and Dickhoefer, 2020).

1.3 Ensiling

In view of the various challenges associated with forage legumes' cultivation and their year-round availability for ruminants' feeding in the tropics, the conservation of forage legumes as silage offers a valuable option, because it is an ideal conservation method that makes forages available for use in periods of feed deficit (Kaiser et al., 2004). Over time, the proportion of forage conserved as silage globally has increased due to improved silage making technology and mechanization of silage feeding systems (Wilkins and Wilkinson, 2015). Similarly, the reduction in the susceptibility of silage to adverse weather and conservation losses compared to hay has contributed to the rise in silage production.

Ensiling is a biochemical process during which simple plant sugars, e.g. glucose and fructose, are converted by lactic acid bacteria (LAB) into lactic acid in an anaerobic fermentation that is governed by certain conditions that are forage and storage-related (Fijałkowska et al., 2015; Rooke and Hatfield, 2003). According to the major biochemical and microbiological transformations that occur during ensiling, ensiling is divided into four phases (Figure 1), which include the intial aerobic period, anaerobic fermentation, stabilization phase and feed-out phase (Rooke and Hatfield, 2003). The choice of forage at ensiling is a critical factor during ensiling because it affects all biochemical and microbiological interactions (Teixeira Franco et al., 2016). Although there is a variation in the ensilability of both legumes and grasses, legumes

are more difficult to ensile than grasses due to their considerably greater buffering capacity and lower concentrations of water-soluble carbohydrates (WSC; Fijałkowska et al., 2015).

In forages rich in CP, like legumes, considerable changes in the concentration and composition of the CP fraction begins at the time of harvest and last till ensiling period, during which extensive hydrolysis of protein occurs (Figure 2) through two pathways (Fijałkowska et al., 2015). The first pathway is under aerobic conditions, during which CP is degraded to non-protein nitrogen compounds (NPN) by proteolytic plant enzymes. The activity of proteases in forage increases at raised temperature conditions in the silo, and extensive hydrolysis of CP to amino acids occur (Dunière et al., 2013). Furthermore, enterobacteria influence the decarboxylation and deamination of amino acids under anaerobic conditions, resulting in the formation of nitrogen oxide, ammonia, and biogenic amines (Rooke and Hatfield, 2003). While the extent of proteolysis in forage is the effect of forage enzymes activities, the breakdown of amino acids is confined by the activity of enterobacteria, species composition of LAB and the increase of proteolytic bacteria (Winters et al., 2001). Thus, the control of biochemical processes and the growth of different microorganisms seems rather important to obtain a good legume silage quality.

Most silage studies have shown a decrease in the CP concentration of legumes prepared as silage compared to the fresh forage due to soluble nitrogen losses via effluents (Albrecht and Muck, 1991; Bureenok et al., 2016; Grabber, 2009; Krawutschke et al., 2013). Similarly, a large concentration of WSC, which is limited in forage legumes compared to grasses, decreases during ensiling, resulting in deficient energy substrates for rumen microbes in ruminants (Fijałkowska et al., 2015). These changes in nutritional concentration during ensiling are dependent on controllable (silage management) and uncontrollable factors (climate-related), which may further affect the efficiency of forage nutrient utilization by ruminants (Bernardes et al., 2018; Broderick, 2018). Thus, it is crucial that the quality of nutrient supply from forage legume silage, particularly protein, a decisive nutrient for improved ruminant production, is assessed and satisfied because it is often a limiting nutrient in ruminant diets in the tropics.

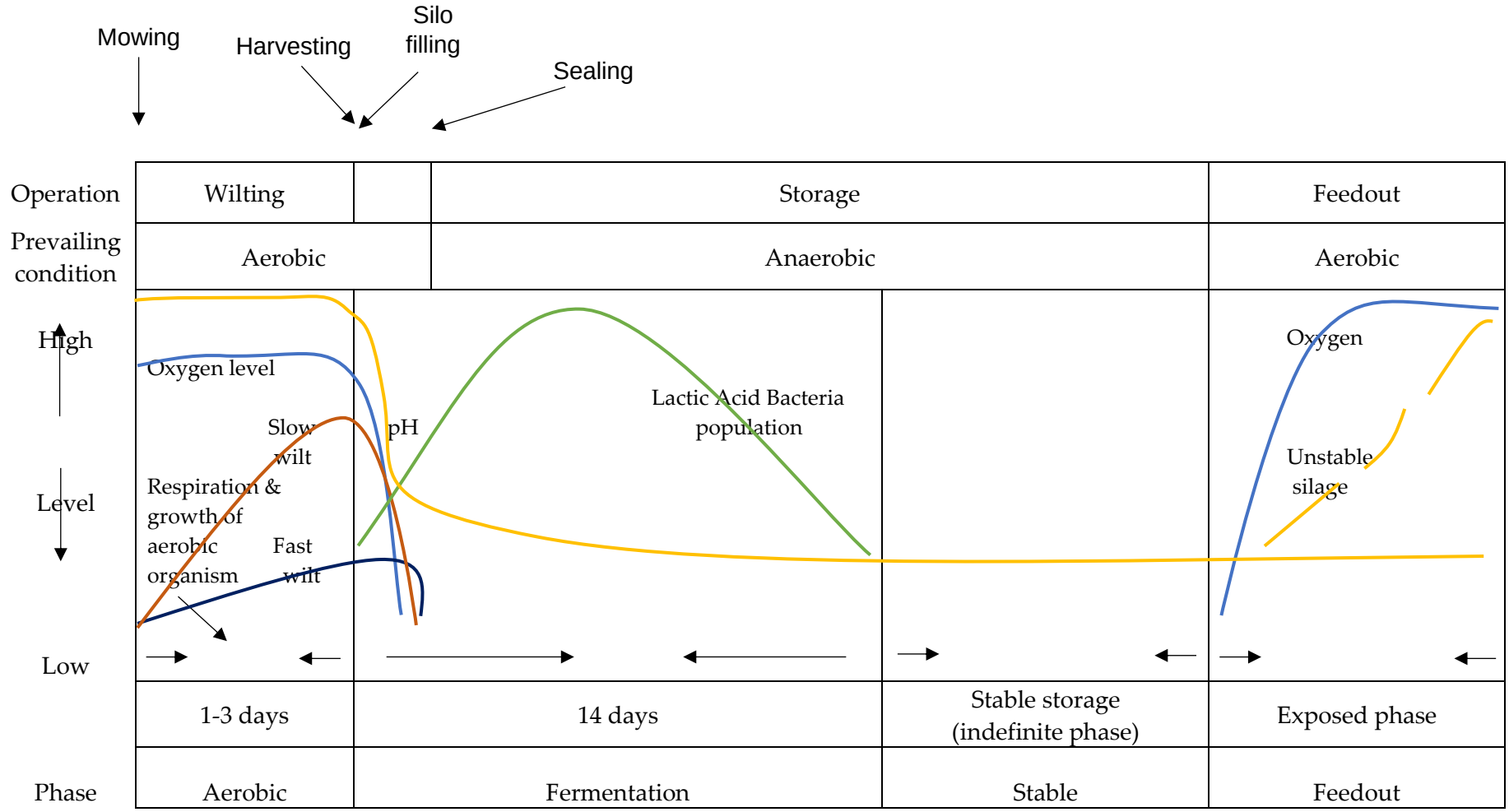
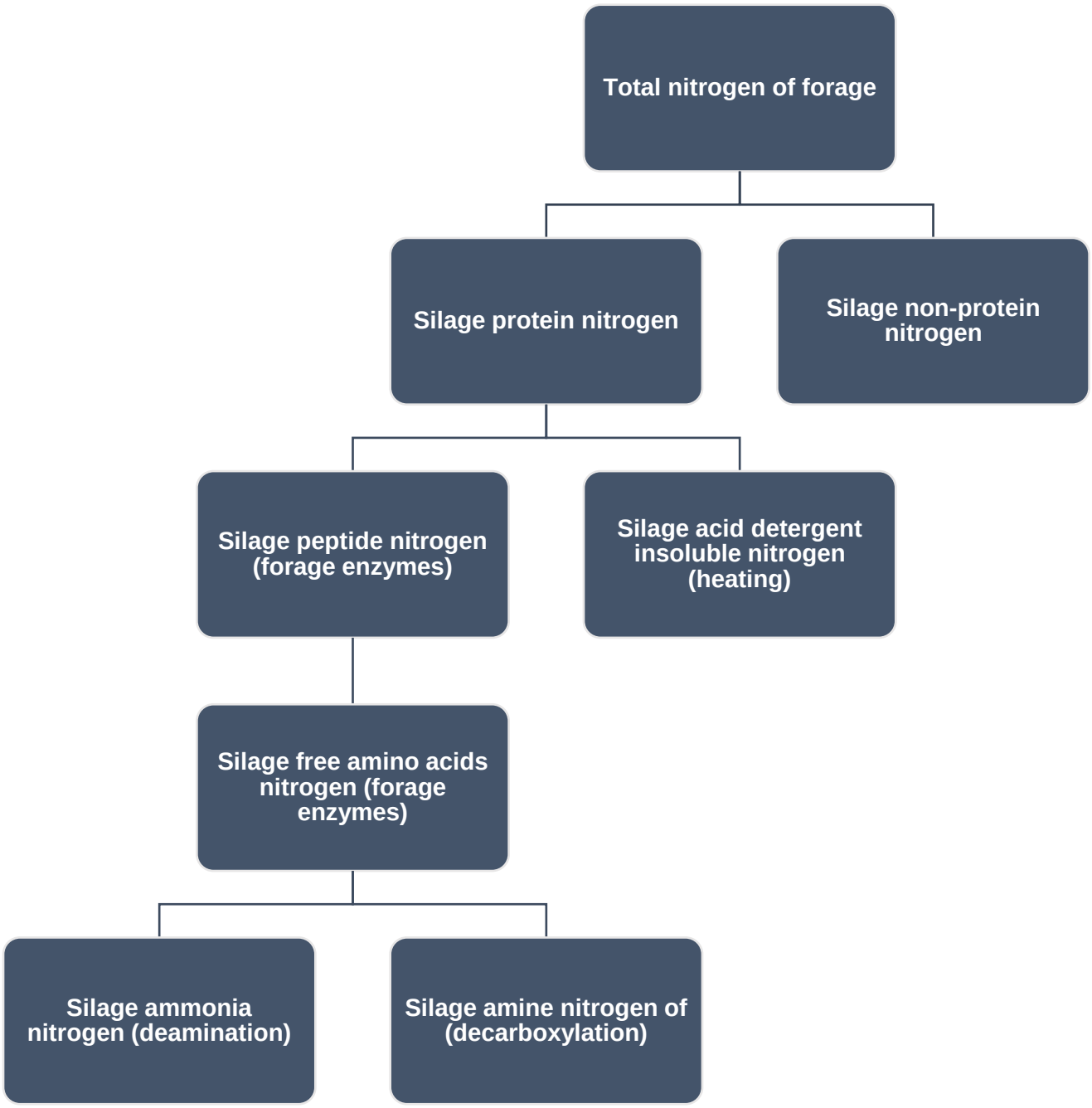


Figure 1. Changes occurring during the various phases of silage production

Source: Adapted from Pitt (1990)



Source: Adapted from Fijałkowska et al., 2015

Figure 2. Composition of total nitrogen of silage.

1.4 Ensiling conditions

In addition to the choice of forage as a critical factor that influences all biochemical and microbiological interactions during the ensiling period are the ensiling conditions. These conditions are mainly storage-related, including the aerobic condition, packing density, storage duration and storage temperature. The addition of fermentation stimulants also falls within the frame. For instance, the seepage of oxygen into the silo prevents the achievement of anaerobic conditions, thereby preventing LAB proliferation. An aerobic stability study by Garcia et al. (1989) on a laboratory scale alfalfa silage with 320 mL/day air rates for 21 days reported lower lactic acid concentration and higher pH and ammonia-nitrogen concentration in aerated silage, an indicator of poor silage quality.

Factors such as ambient temperature (Muck and Dickerson, 1988) and ensiling length, which allows for prolonged microbial processes (Kleinschmit and Kung, 2006), are still the main challenge to ensure good silage quality. Under a prolonged ensiling period of alfalfa, it has been reported that increased ensiling length increases the concentration of crude protein (CP), soluble protein and ammonia-nitrogen with no changes in the fibre concentrations after 360 d of ensiling (Santos and Kung, 2016). Besides, decreased neutral detergent fibre (NDF) concentration and increased proteolysis after 365 d of ensiling corn was reported (Der Bedrosian et al., 2012; Herrmann et al., 2011). However, in these studies, silages were stored at a room temperature of about 23°C, and high temperatures can alter microbial activities during ensiling (McDonald et al., 1966). Significant temperature variations are expected within the silo regardless of the ensiling location because the silo is usually submitted to ambient temperature (Teixeira Franco et al., 2016). For instance, Garcia et al.(1989) reported an increase in the concentration of acid detergent insoluble nitrogen (ADIN) and fibre constituents of alfalfa silage stored for 21 d at 38 and 65°C with increasing temperature. Moreover, the study of Kim and Adesogan (2006) with corn silage at 20 and 40 °C for 82 days showed higher residual WSC, pH and ammonia-nitrogen concentration and lowered lactic to acetic acid ratio at the highest temperature. This finding suggests that, at 40 °C, fermentation was more lengthy, reflecting reduced silage quality at the end. Thus, ensuring optimal ensiling conditions seems rather important to produce high-quality silage.

1.5 Forage legume silage and ruminant nutrition

Silages are an essential source of nutrients in ruminant nutrition. The acceptability of legume silages by ruminants is greater than that of grass silages, and legume silages tend to increase animal performance (Dewhurst, 2013; Steinshamn, 2010). Even at similar or lower total tract digestibility compared to grass silage, the faster digestion rate in legume than grass silage can result in higher energy and protein intakes by ruminants (Buxton and Redfearn, 1997; Phelan et al., 2015). However, the utilization of nutrients of tropical legume silages when included in ruminant diets is inconsistent compared to temperate legumes. According to a recent meta-analysis of 52 studies on the effects of including tropical legume silage in ruminant diets, feed intake and nutrient digestibility were reduced at inclusion levels below 400 g/kg dry matter (DM) of legume silages in ruminant diets. Still, the effect of including tropical legume silage on milk yield and average daily gain either remains the same or increases slightly compared to diets without legume silage or diets with legume silage above 400 g/kg DM (Castro-Montoya and Dickhoefer, 2018). Undoubtedly, these findings negate the theory that less feed intake increases digestibility and nutrient utilization in ruminants. In addition, this indicates that not all attributes given to temperate legume silages, like higher feed intakes and nutrient digestibilities compared to grass silages or whole crop silages, can be adopted for tropical legume silages (Castro-Montoya and Dickhoefer, 2018). Given that ensiling conditions play vital roles in legume silage quality, the effects of diets including legume silages on ruminants' performance cannot be totally isolated from the processes that occur during ensiling.

As the storage length of silage on many farms may exceed several months and the temperature in the tropics is characteristically high, changes in the nutritional value of forage legume silage are imminent. Besides, the activity of legume forage proteases increases and extensive hydrolysis of proteins to amino acids and further deamination to ammonia and amines occur under prolonged high storage temperature (Fijałkowska et al., 2015). Consequently, feeding legume silages under prolonged high storage temperature may modulate rumen fermentation due to loss of fermentable substrates (protein and energy) during prolonged ensiling, which may render those nutrients unavailable for ruminants. Along this line, supplementing the deficient nutrient (protein and energy) as a

result of ensiling conditions might be a valuable option to achieve the desired performance in ruminants.

1.6 Scope of the thesis

Tropical silage research mostly focuses on assessing the silage's chemical composition without considering the effect of ensiling conditions on silage quality and the resultant effect on ruminant performance. Yet, the assessment of the chemical composition of silages alone is not a sufficient predictor of the nutritional value because the nutritive value is a product of nutrient availability and the efficiency of nutrients derived from feed independent of intake (D'Mello and Devendra, 1995). The aim of this thesis was to understand the changes in the nutritional quality of forage legumes in the tropics when ensiled under different conditions and the impact of feeding such legume silages on rumen fermentation and nutrient utilization in tropical cattle.

To achieve this aim:

- A first study (Chapter 2) was conducted to evaluate the effect of ensiling length and storage temperature on the nutritional value, fermentation characteristics, and concentration of fibre-bound protein in three tropical legumes ensiled solely or in combination with sorghum forage. The hypothesis tested was that high temperatures in the silages associated with outdoor storage in the Tropics together with long durations of storage of silages would slow down fermentation activity and increase fibre concentration and fibre-bound nitrogen of legume forages ensiled alone or in combination with sorghum forage.
- In the second study (Chapter 3), the *in vitro* ruminal fermentation and post-ruminal digestibility of sorghum-soybean forage as affected by ensiling length, storage temperature and CP levels were assessed. The hypothesis was that *in vitro* ruminal fermentation would be affected differently by the studied factors with higher nutrient degradability at increased incubation time. Additionally, storage temperature and ensiling length will reduce the post-ruminal digestibility of CP with higher digestibility of high CP diets.

- The final study (Chapter 4) evaluated the effect of two levels of dietary CP concentrations on nutrient digestibility, performance, nitrogen metabolism and blood metabolites of tropical growing steers fed corn or corn-soybean silage. The hypothesis was that feeding corn-soybean silage with low CP will be more efficient by increasing microbial protein synthesis and growth, while corn silage fed with high CP will be less efficient with reduced microbial protein synthesis and growth.

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Chapter 2

2. Effects of ensiling length and storage temperature on the nutritive value and fibre-bound protein of three tropical legumes ensiled alone or combined with sorghum

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2.1 Abstract

Changes in the nutritive value of forages are imminent under different ensiling conditions. Thus, a study was conducted to assess the effects of ensiling length and storage temperature on the nutritive value, fermentation characteristics and fibre-bound protein of three tropical forage legumes, sorghum and mixtures of sorghum and the legumes. Soybean (*Glycine max*), jack bean (*Cannavalia ensiformis*), lablab (*Lablab purpureus*) and sorghum (*Sorghum bicolor)* were solely grown and harvested, and the legumes were wilted before ensiling. Mixtures of sorghum and each legume were handmade on a percentage fresh weight basis of 60:40. Each forage and mixtures (400 g) were ensiled in polythene vacuum bags with homofermentative lactic acid bacteria inoculation for 30, 75 and 180 days. A set of mini silos were stored indoors, and another batch was stored outdoors. HOBO Pro v2 data loggers were deployed to monitor the ambient temperature of the storage locations during the entire ensiling period (from day 0 to 180). Measurements included nutrient analysis, fermentation quality and fibre bound protein characteristics. The hourly ambient temperature for outdoor and indoor storage ranged from 16-61°C vs 18-35°C, respectively. Proximate constituents of all silages were influenced by ensiling length. Significant changes were primarily detected in fermentation products of legume silages between 30 and 75 d of ensiling. There were reduced fermentation products for silages stored outdoors. The ensiling length influenced proportions of neutral detergent insoluble nitrogen (NDIN) and acid detergent insoluble nitrogen (ADIN) with outdoor silages resulting in a higher proportion of NDIN and ADIN compared to indoor silages. Overall, a short period of ensiling preserves the nutritional quality of ensiled forages compared to prolonged storage at high ambient temperatures typical of the tropics that increase nutrient losses. Thus, changes in the nutritional composition of forages during ensiling should be considered during ration formulations.

KEYWORDS: ensiling, temperature, sorghum, lablab, soybean, jack bean

2.2 Introduction

Silage production is an ideal conservation method that offers a valuable option of addressing feed shortage in times of deficit with high-quality feed from biomass harvested

during the growing period (Kaiser *et al.*, 2004). Recently, the proportion of forage conserved as silage has increased in the tropics (Wilkins and Wilkinson, 2015), where clear differences in forage availability are found between the rainy and dry seasons. However, during the ensiling process, there are inevitable changes in the nutritional composition of the ensiled forage compared to the fresh forage, and this depends on the species, cultivars of forage for silage (Pichard *et al.*, 2006) and the ensiling conditions. With legume forages, the rise in silo temperatures causing Maillard reactions often result in significant proteolytic activities leading to significant changes in crude protein (CP) fractions which may reduce the efficiency of forage nitrogen utilization by ruminants (Bernardes *et al.*, 2018; Broderick, 2018).

The wilting of forage prior to ensiling as well as the use of silage additives have shown potential to minimise proteolysis and Maillard reactions during ensiling (Kaewpila *et al.*, 2021; Muck *et al.*, 2018; Owens *et al.*, 1999). However, ambient temperature and ensiling length directly affect microbial processes and, consequently, crude protein degradation (Muck and Dickerson 1988; Kleinschmit and Kung, 2006). Prolonged ensiling length increased the concentration of crude protein (CP), soluble protein and ammonia-nitrogen with no changes in the fibre concentrations of alfalfa after 360 d of ensiling (Santos and Kung, 2016). In contrast, decreased neutral detergent fibre (NDF) concentration and increased proteolysis was reported in corn silage after 365 d of ensiling (Der Bedrosian *et al.*, 2012; Herrmann *et al.*, 2011).

Storage temperature has been found to affect microbial activities during ensiling (McDonald *et al.*, 1966). Garcia *et al.* (1989) reported an increase in the concentration of acid detergent insoluble nitrogen (ADIN) and fibre constituents of alfalfa silage when the temperature increased from 38 to 65°C. Nonetheless, all these studies were conducted in the temperate, and the conditions differ from the tropics. Moreover, the leaf and stem anatomy and morphological characteristics of temperate and tropical legumes differ despite having the same metabolic pathway C_3 of carbon fixation (Muir *et al.*, 2014). There is the likelihood of microbial processes in silo differs.

Most studies on silages from tropical forage legumes still centred on defining the nutrient concentrations and silage fermentation quality, but less is dedicated to how the process of

ensiling affects the final silage's nutritional quality, neither the underlying processes. It has been reported that tropical legume silages cannot assume all attributes given to temperate legume silages (Castro-Montoya and Dickhoefer, 2018). This finding cannot be isolated from the forage characteristics and ensiling processes in which the preharvest and ensiling factors play key roles. The majority of silage studies involving tropical forages were evaluated within 30 and 92 days after ensiling (Heinritz *et al.*, 2012; Li *et al.*, 2019; Lima-Orozco *et al.*, 2014; Nkosi *et al.*, 2016) and storage conditions ranging between 25 - 40°C. These storage conditions do not reflect typical field realities because the storage of silages on farms may exceed six months, considering the long dry seasons typical of the tropics. It is expected that forage legumes ensiling under a prolonged period and at high tropics temperatures would affect microbial processes, thereby increasing fibre-bound protein proportion. Thus, the present study's objective was to study the effects of storage temperature on the nutritive value, fermentation end-products and fibre-bound protein of forages from soybean, jack bean, lablab, sorghum and the legumes' mixture with sorghum after various lengths of storage in silo.

2.3 Materials and methods

2.3.1 Field Management

The experimental field was situated at the University of El Salvador research station in San Luis Talpa (13°28′19″N, 89°05′59″W, at 62 m.a.s.l.) in a relatively levelled and well-drained terrain of sandy loam (brown soil). Soil tillage operations prior to the establishment of the crop at the location comprised disc ploughing and harrowing.

2.3.2 Forages

Seeds sown for this experiment were obtained from Centro Nacional de Tecnología Agropecuaria (CENTA), El Salvador. Three herbaceous legumes (soybean [*Glycine max*, SIATSA-194], jack bean [*Canavalia ensiformis*, CENTA], and lablab [*Lablab purpureus*, CENTA]) and sorghum (*Sorghum bicolor*, CENTA RCV) were used in the present study. The seeds of jack bean, lablab and sorghum were hand sown during the rainy season as a sole crop in a randomized complete block design arrangement with four replications (i.e., plots) on 16th May 2018, while soybean was sown similarly on 25th May 2018 to synchronize

harvesting of plots at the desired maturity stage. Each plot measured 4 m long and 3.2 m wide with five rows (0.8 m spacing) and 1.5 m alleys partitioning the plots. Before planting, seeds were treated with Blindage (imidacloprid-thiodicarb), a seed protectant (Bayer AG GmbH, Leverkusen, Germany). The rate of seeding was aimed to attain a plant density of 150,000 plants/ha for sorghum, jack bean, and lablab respectively and 225,000 plants/ha for soybean. Weed control was performed manually, and each plot received 100 kg/ha of ammonium sulphate fertilizer in bands along the rows during seeding. Monarca (thiacloprid, beta-cyfluthrin) was also applied as required to control pests. Irrigation was provided during a 3-weeks-dry period approximately six weeks before harvest. Harvesting was done manually in the early hours on a sunny day at the flowering stage for jack bean and lablab (88 days post sowing), and 79 days post sowing for soybean at the R3 stage. However, sorghum was harvested at the mid-dough stage after 6 h of harvesting the legumes' plot. All forages were harvested to a 5-cm-stubble height from the three middle rows of a net plot area of 4.86 m^2 from each subplot. Samples (~ 500 g fresh matter) of fresh forages were chopped and immediately frozen and stored at -20°C in triplicate for further analyses.

2.3.3 Ensiling

The legume forages were wilted outdoors for 6 h after harvest, whereas sorghum forage was not wilted before chopping. Subsamples (400 g) of sorghum fresh forage and wilted legumes in triplicates were frozen forthwith at -20°C for later analysis after chopping to a particle size of 13 mm with a precision forage chopper (JF Máquinas, Itapira, Brazil). The chopped forage was homogenized and inoculated with sila-prime (Star-Labs, Clarksdale, USA). In consideration of the high buffering capacity of forage legumes, homofermentative lactic acid bacteria (LAB) was added. The addition of homofermentative LAB was due to its faster rate of lactic acid production during silage fermentation which will lower the pH at a faster rate (Muck *et al.*, 2018). According to the manufacturer, sila-prime contains the homofermentative LAB *Lactobacillus plantarum*, *Pediococcus acidilactici*, and *Pediococcus pentosaceous*. The inoculant (4.5 g) was dissolved in 1 L tap water and applied evenly at a rate of 2 mL/kg fresh matter to the chopped forage using a pressure sprayer. In addition to the sole sorghum and legumes forage, three mixtures of sorghum with each of soybean

(sorghum-soybean), jack bean (sorghum-jack bean), and lablab (sorghum-lablab) were prepared at sorghum (fresh matter) to legume (wilted) ratio of 60 to 40 (on fresh matter basis). After thorough mixing, 400 g fresh matter of each inoculated chopped forage and their mixtures were packed in embossed polythene vacuum bags (252 bags in total) with continuous fluted structure (170 µ thickness, 15 cm x 30 cm; Allpax GmbH, Papenburg, Germany) and heat-sealed with an Allpax vacuum machine (Allpax GmbH, Papenburg, Germany). Before sealing, the vacuum machine extracted 97% of the air from the bag, according to the manufacturer.

2.3.4 Study factors

Two factors were studied: the effect of storage temperature (i.e., outdoor vs indoor) and ensiling length (i.e., 30, 75, and 180 days). For storage temperature, a set of silages was stored outdoors on the roof of a building under direct sunlight (outdoor storage). The second set of silages was stored indoors at room temperature in a ventilated laboratory room (indoor storage). All silos were stored in plastic totes with lids that were covered with the black plastic sheets which are commonly used in trench silos on the field. Two HOBO Pro v2 temp/RH data loggers (Onset Computer Corporation, Bourne, USA) were deployed during the ensiling period to monitor the temperature within each plastic tote logging temperature at 1 h intervals. The data logger was positioned at the centre of the plastic tote with a dimension of 75 cm × 55 cm × 47 cm. Moreover, for both storage conditions, silages were conserved for ensiling lengths of 30, 75, and 180 days. Additional silos were prepared to monitor changes in pH at 3, 9, and 16 days in the initial phase of ensiling. All forage × temperature × length of ensiling combinations were ensiled in triplicates for a total of 252 laboratory-scale silos (i.e., 7 forages x 2 temperatures x 6 ensiling lengths x 3 replicates).

2.3.5 Sampling and processing

Sampling was done freshly at harvest (for all forages), after wilting (for soybean, jack bean, and lablab) and after opening the silos at 3, 9, 16, 30, 75, and 180 d. At the end of 30, 75 and 180 d ensiling length, samples of 300 g fresh matter of the silages were taken, thoroughly mixed, and placed in a transparent plastic 20 cm x 30 cm pouch for storage at -20°C for later analysis.

All samples were taken for pH measurement, whereas samples from silages at 30 d and 75 d were processed for ammonia-nitrogen (NH_3-N), lactic acid and acetic acid concentrations. For analysis of fermentation parameters, an extract of samples was prepared by mixing 10 g of the frozen samples with 100 mL distilled water, blending the mixture for 30 s, and filtering it through two layers of cheesecloth. The pH of the extract was measured directly using a pH meter (WTW Multi 340i, WTW, Weilheim, Germany). Aliquots (10 mL) of the extract from ensiled samples were then centrifuged at 18,000 × *g* for 10 min, and the decanted supernatant was stored at -20°C for lactic and acetic acid analysis. Additionally, 2 mL of the extract was deproteinized with 6 mL of trichloroacetic acid (0.3 M) and allowed to stand at room temperature for 2 h before centrifugation at 18,000 × *g* for 10 min. The supernatant obtained was used for the analysis of NH_3-N.

2.3.6 Chemical analysis

All samples were analyzed in duplicate. The remaining sample material (290 g fresh matter) were initially dried in a forced-draught oven at 60°C for 48 h. Dried samples were ground through a 1-mm-screen in a Wiley mill (Thomas Scientific, Swedesboro, USA) and stored for further analysis. The DM concentration of samples was further determined by drying them at 103°C for 24 h in a forced-draught oven (VDLUFA, 2012), and volatile losses in silage samples were corrected according to Weißbach and Strubelt (2008) equation:

$$\text{DMcorr (g/kg fresh matter)} = 26.2 + 0.97 \times \text{UDM} \quad (1)$$

where DMcorr is the corrected DM concentration and UDM is the uncorrected DM content of the silage samples.

Neutral detergent fibre (aNDF; assayed with heat-stable amylase and sodium sulphite) and acid detergent fibre (ADF) concentrations (both inclusive ash residues) were determined sequentially using an ANKOM 200 fibre analyzer (ANKOM Technology, New York, USA) following the methods of VDLUFA (2012). Total nitrogen (N) concentration was determined by Dumas combustion using vario MAX CN element analyzer (Elementar Analysensyteme GmbH, Hanau, Germany) and crude protein (CP = N × 6.25) was calculated thereof. Additionally, crude ash (CA), crude lipid (CL) and acid detergent fibre after ashing (ADFom) were determined according to VDLUFA (2012) for treatment samples used for metabolizable energy (ME) and digestibility of organic matter (DOM) estimation.

For the estimation of the slowly degraded CP fraction (B_3) in the Cornell Net Carbohydrate Protein Model (CNCPS), NDIN and ADIN concentrations were determined following the standardization procedures for nitrogen fractionation (Licitra *et al.*, 1996) without sodium sulphite application. For NDIN determination, grounded samples of fresh, wilted and ensiled forage (1 g) were boiled in 100 mL of neutral detergent solution with the refluxing process for 1 h. Approximately 16 µL of alpha-amylase (ANKOM Technology, New York, USA) was added after 10 min and 30 min of boiling. Then, the boiled sample solution was filtered with suction on weighed filter paper (Whatman paper N° 54, GE Healthcare Life Sciences, Darmstadt, Germany), which had previously been dried at 103°C for 2 h. About 250 mL of hot distilled water (80°C) was used to soak and wash the sample residue and filter paper before rinsing them twice with 10 mL of acetone. Consequently, sample residue in folded filter paper was air-dried for 3 h at room temperature, oven-dried at 103°C for 2 h, and cooled in a desiccator for at least 20 min before transferring the samples to Kjeldahl flasks for N analysis. The ADIN analysis followed the same procedure using acid detergent instead of neutral detergent solution and without adding alpha-amylase. The NDIN and ADIN were presented as a proportion of crude protein (g/kg CP). Afterwards, B_3 was calculated by subtracting ADIN from NDIN proportion.

For metabolizable energy (ME) and digestibility of organic matter (DOM) estimation, 50 samples in total were incubated in view of examining only the effect of ensiling length. One sample each was selected from the triplicate dried fresh, wilted and ensiled samples of all treatment and was incubated in the Hohenheim Gas Test system for 24 h (Menke and Steingass, 1988) to measure the cumulative gas production (GP). Each sample was incubated in two runs with three replicates within each run. The ME and DOM were estimated using the following equations (GfE, 2016):

$$ME^1 = 12.49 – (0.0114 \times ADFom) + (0.00425 \times CP) + (0.0269 \times CL) + (0.01683 \times GP) \quad (2)$$

with ME^1 in MJ/kg OM; CP, CL, ADFom in g/kg OM; and GP in mL/200 mg OM

$$ME^2 \text{ (MJ/kg DM)} = ME^1 \times (1000 – CA) / 1000 \quad (3)$$

with ME^2 in MJ/kg DM; ME^1 in MJ/kg OM; and CA in g/kg DM

$$DOM = 95.72 – (0.0859 \times ADFom) + (0.0964 \times GP) \quad (4)$$

with DOM in g/100 g; ADFom in g/kg OM; and GP in mL/200 mg OM.

In the present study, fermentation analysis was only carried out for silage samples of 30 and 75 d ensiling period due to insufficient fermentation assay kits. The concentration of lactic acid, acetic acid and NH_3-N of ensiled samples were analyzed using the megazyme enzymatic procedure (Megazyme International, Wicklow, Ireland) with the assay kits K-DLATE, K-ACET and K-AMAIR, respectively. The reagents for each kit were prepared as specified procedurally, and the manual assay of the megazyme procedure was adopted. At a ratio 1:10, prepared silage extract was diluted with distilled water. The buffer stock and prepared reagent solutions of K-DLATE or K-ACET were added measurably to the diluted silage extract and blank sample (distilled water) in a stepwise manner. After thorough mixing using a vortex, the mixed solution's absorbance was read twice for lactic acid and thrice for acetic acid determination at 340 nm using a spectrophotometer (Jenway 6305Jenway, Staffordshire, UK). Subsequently, the silage samples' lactic or acetic acid concentrations were calculated after the absorbance difference of the two or three readings of the blank and silage samples were determined using the megazyme calculation excel sheet. For the determination of NH_3-N concentration, the buffer stock and prepared reagent solutions of K-AMAIR assay were added to pure silage extract and blank sample (distilled water) step by step, and the mixed solutions were thoroughly mixed using a vortex. The absorbance of the mixture was read twice at 340 nm using the same spectrophotometer. After that, the absorbance difference of the two readings of the blank and silage samples was used to calculate the NH_3-N concentration using the megazyme calculation excel sheet.

2.3.7 Statistical analysis

For variables on nutrient concentrations and fibre-bound nitrogen, a two-by-four factorial design (storage temperature (outdoor, indoor), ensiling length (0, 30, 75 and 180 d)) and their interaction with three replicates per treatment per forage was conducted with the GLM procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC, USA) using the following model:

$$Y_{ij} = \mu + T_i + L_j + (TL)_{ij} + e_{ij}$$

where Y_{ij} = dependent variable, μ = overall mean, T_i = temperature effect, L_j = ensiling length effect, $(TL)_{ij}$ = the interaction effect of temperature and ensiling length and e_{ij} = residual random error of experiment.

The analysis for the variables of fermentation characteristics followed the same model as described before but with a two-by-two factorial design (storage temperature (outdoor, indoor), ensiling length (30 and 75 d)) and their interaction with three replicates per treatment per forage.

Pre-ensiled forage characteristics were analyzed using the model:

$$Y_i = \mu + F_i + e_i.$$

where Y_i = dependent variable, F_i = forage effect, μ = overall mean, and e_i = residual random error of experiment.

Changes in pH were determined using a 2-storage temperature (i.e., outdoor, indoor) × 6-ensiling lengths (0, 3, 9, 16, 30, 75, 180) factorial design.

One-way ANOVA was conducted to compare the effect of only ensiling length on the data obtained from ME and DOM estimates using the model:

$$Y_i = \mu + L_i + e_i.$$

where Y_i = dependent variable, L_i = effect of ensiling length, μ = overall mean, and e_i = residual random error of experiment.

Linear, quadratic and cubic effects of ensiling length were determined using orthogonal polynomial contrasts for nutrient concentrations and fibre-bound nitrogen variables.

Tukey-Kramer test was used to compare treatment least-square means for variables of pre-ensiled forage, and the significance level was declared at $P < 0.05$.

2.4 Results

2.4.1 Temperature measurement

Across each hourly measurement, the outdoor storage temperatures ranged from 16°C to 61°C as against 18°C to 35°C recorded for indoor temperatures during the study period between August 2018 and February 2019 (Figure 1). The equivalent of the sum of hourly temperatures in days that were above 30°C was 72 days for outdoor and 1 day for indoor storage. On a daily basis, the average and maximum temperature recorded was 30°C and 37°C for outdoor and 25°C and 28°C for indoor, respectively.

Table 1: Chemical composition (in g/kg DM or as stated) of pre-ensiled wilted legume and fresh sorghum forage (least square means and standard error of means (SEM), n = 3)

Variable	Soybean	Jack bean	Lablab	Sorghum	SEM	*P*-value
DM (g/kg FM)	351[a]	266[b]	236[b]	226[b]	10.2	<0.01
Ash	85.9[b]	114[a]	122[a]	88.9[b]	2.24	<0.01
CP	148[b]	196[a]	179[a]	98.8[c]	5.21	<0.01
aNDF	427[b]	451[b]	495[ab]	529[a]	15.6	<0.01
ADF	292[b]	305[ab]	352[a]	281[b]	12.5	<0.05
NDIN (g/kg CP)	55.3[b]	62.7[a]	63.0[a]	50.4[c]	0.75	<0.01
ADIN (g/kg CP)	17.2[b]	22.1[a]	26.4[a]	13.9[b]	1.02	<0.01
B_3 (g/kg CP)	38.1	40.1	36.6	36.5	1.16	n.s
ME (MJ/kg DM)	9.0	8.6	7.8	9.7	-	-
DOM (g/100 g OM)	74.2	73.3	66.9	79.2	-	-

[a–c] Means within rows with different superscripts differ ($P < 0.05$); n.s., not significant

DM, dry matter; FM, fresh matter; CP, crude protein; aNDF, neutral detergent fibre with alpha amylase; ADF, acid detergent fibre, NDIN, neutral detergent insoluble nitrogen; ADIN, acid detergent insoluble nitrogen; B_3, slowly degraded CP fraction; ME, metabolizable energy and; DOM, digestibility of organic matter

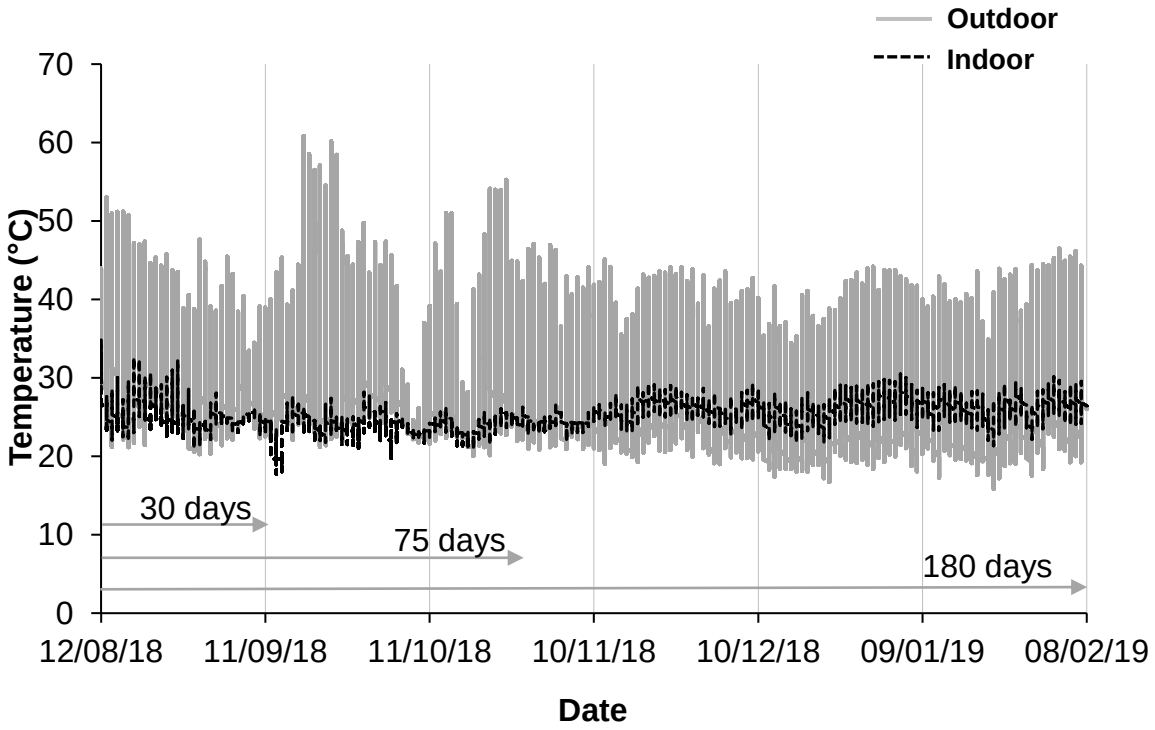


Figure 1. Hourly diurnal variation of outdoor and indoor temperature at various ensiling lengths.

2.4.2 *Pre-ensiled forage characteristics*

Among the pre-ensiled forages, the DM concentration was greatest for soybean ($P < 0.01$; Table 1). Crude protein concentration was greatest for jack bean and lablab and lowest for sorghum ($P < 0.01$). The concentration of aNDF was greater in sorghum ($P < 0.01$) than in all the legumes, which did not differ from each other. The ADF concentration was highest in lablab, followed by jack bean, soybean and the lowest concentration was found in sorghum ($P < 0.01$). Lablab and jack bean had the highest proportion of both NDIN and ADIN, followed by soybean, whereas it was lowest in sorghum ($P < 0.01$). No significant differences were found between the forages for the proportion of the CP fraction B_3. The ME concentrations and DOM were numerically greatest in sorghum and lowest in lablab when compared to one another (no statistical analysis could be performed because n = 1 for these parameters as earlier stated).

2.4.3 *Chemical composition of silages*

In most cases, storage temperature did not affect DM, CP, aNDF, and ADF concentrations, while there were quadratic and cubic effects of ensiling length on those variables (Table 2). No ensiling length × temperature interaction was observed. In all silages, DM concentration increased through 75 d of ensiling before declining ($P < 0.01$). The decrease in DM concentration after 75 d of ensiling was less in sole legumes compared to mixed sorghum-legume silages. The ensiling length did not affect CP concentration in sorghum silage, but there was a cubic effect on soybean and soybean-sorghum silages, with maximum values at 30 and 75 d of ensiling. Similar effects were observed for lablab silages, but there was quadratic effect in CP concentration with increasing ensiling length for jack bean – both, ensiled alone and mixed with sorghum. The aNDF and ADF concentration behaved mostly opposite to that of CP, where soybean, lablab, and sorghum-lablab showed their lowest concentration through 30 d and increased at 180 d. Sorghum and sorghum-soybean silages showed the highest drop at 75 d, while jack bean and sorghum-jack bean silages showed rather a substantial increase at 180 d.

Storage temperature only affected the CP concentration of sorghum-jack bean ($P < 0.01$) and the aNDF concentration of the lablab silage ($P < 0.05$) with higher concentrations observed in outdoor samples compared to indoor.

2.4.4 *Metabolizable energy and digestibility of organic matter*

The concentrations of ME and DOM in soybean and lablab silages were greater at 75 d than 30 d and 180 d of ensiling, whereas, in jack bean silage, ME was lower at 180 d, and DOM was lower for all ensiling lengths than in the fresh forage ($P < 0.01$ for all variables and comparisons). No effect of ensiling length was recorded on the ME and DOM concentrations of sorghum silages (Table 3). The ME concentration of sorghum-soybean silage was lowest at 180 d of ensiling ($P < 0.05$). Concentrations of ME and DOM in sorghum-lablab only declined after 75 d ($P < 0.05$), while there was a decrease from d 0 to d 180 in sorghum-jack bean ($P < 0.01$)

2.4.5 *Fermentation characteristics*

Initial pH of all silages ranged from 5.87 to 7.78, declining rapidly until the 3rd day of ensiling and stabilizing in most silage till the 180th day at a pH ≤ 5 (Figure 2). Final pH after 180 d of ensiling of all sole legume silages (4.61) was greater than that of sorghum (3.81) and mixed sorghum-legume silages (3.97). Interaction of ensiling length and storage temperature was observed in pH of soybean, sorghum, sorghum-soybean, sorghum-jack bean and sorghum-lablab ($P < 0.01$). In most silages with interaction effect, the greatest difference in pH between outdoor and indoor storage was found on 9 d as pH decrease with ensiling length except for soybean silage, with the greatest difference in pH found on 180 d. The pH in all silages stored outdoors was greater than those stored indoors. Additionally, the pH of

Table 2. Effect of ensiling length, ambient temperature, and their interaction on the chemical composition (in g/kg DM or as stated) of three tropical forage legumes, sorghum and mixed silages (least square means and standard error of means (SEM), n = 3)

Variables	Forages	Ensiling length				Temperature		SEM	*P*-value			Contrasts		
		0 d	30 d	75 d	180 d	OD	ID		L	T	L*T	Lin	Quad	Cub
DM (g/kg FM)	Soybean	351	378	391	363	378	376	15.7	n.s	n.s.	n.s.	n.s.	<0.05	n.s.
	Jack bean	266	303	311	316	315	304	6.50	<0.01	n.s.	n.s.	<0.01	<0.01	<0.05
	Lablab	236	263	275	277	276	267	4.49	<0.01	n.s.	n.s.	<0.01	<0.01	n.s.
	Sorghum	226	247	278	262	270	255	8.01	<0.01	n.s.	n.s.	<0.01	<0.01	n.s.
	Sorghum - soybean	275	303	369	318	333	327	9.12	<0.01	n.s.	n.s.	<0.01	<0.01	<0.01
	Sorghum - jack bean	242	273	322	286	298	289	5.80	<0.01	n.s.	n.s.	<0.01	<0.01	<0.05
	Sorghum - lablab	230	254	281	264	269	263	4.61	<0.01	n.s.	n.s.	<0.01	<0.01	n.s.
CP	Soybean	148	176	170	155	165	168	5.74	<0.01	n.s.	n.s.	n.s.	<0.01	<0.01
	Jack bean	196	177	166	157	165	168	3.90	<0.01	n.s.	n.s.	<0.01	<0.01	n.s.
	Lablab	179	189	194	176	186	186	3.77	<0.01	n.s.	n.s.	n.s.	<0.01	n.s.
	Sorghum	98.8	98.2	95.7	96.4	94.6	98.9	3.51	n.s	n.s.	n.s.	n.s.	n.s.	n.s.
	Sorghum - soybean	119	138	143	132	136	139	2.62	<0.01	n.s.	n.s.	<0.05	<0.01	<0.05
	Sorghum - jack bean	138	136	140	125	128	139	2.62	<0.01	<0.01	n.s.	<0.01	<0.01	n.s.
	Sorghum - lablab	131	143	136	133	136	139	2.73	<0.01	n.s.	n.s.	n.s.	<0.05	<0.01
aNDF	Soybean	427	407	417	508	459	428	20.9	<0.01	n.s.	n.s.	<0.01	<0.05	n.s.
	Jack bean	451	465	485	559	505	500	9.88	<0.01	n.s.	n.s.	<0.01	n.s.	n.s.
	Lablab	495	445	452	515	483	457	10.1	<0.01	<0.05	n.s.	<0.01	<0.01	0.02
	Sorghum	529	492	419	497	475	463	16.6	<0.01	n.s.	n.s.	n.s.	<0.01	n.s.
	Sorghum - soybean	488	449	433	480	460	448	8.94	<0.01	n.s.	n.s.	n.s.	<0.01	n.s.
	Sorghum - jack bean	497	477	472	542	499	494	7.31	<0.01	n.s.	n.s.	<0.01	<0.01	n.s.
	Sorghum - lablab	515	467	481	521	491	487	5.67	<0.01	n.s.	n.s.	<0.01	<0.01	<0.01
ADF	Soybean	292	294	296	376	332	311	21.0	<0.01	n.s.	n.s.	<0.01	n.s.	n.s.
	Jack bean	304	348	368	430	382	381	7.87	<0.01	n.s.	n.s.	<0.01	n.s.	<0.05
	Lablab	352	333	333	382	351	347	8.11	<0.01	n.s.	n.s.	<0.01	<0.01	n.s.
	Sorghum	281	281	241	290	271	270	10.7	<0.01	n.s.	n.s.	n.s.	<0.01	<0.05
	Sorghum - soybean	286	283	270	311	290	285	7.35	<0.01	n.s.	n.s.	<0.01	<0.01	n.s.
	Sorghum - jack bean	291	301	305	363	320	325	4.66	<0.01	n.s.	n.s.	<0.01	<0.01	n.s.
	Sorghum - lablab	310	295	305	340	311	316	5.02	<0.01	n.s.	n.s.	<0.01	<0.01	<0.05

($P < 0.05$); n.s., not significant; L, length; T, temperature; Lin, linear; Quad, quadratic; Cub, cubic; OD, outdoor; ID, indoor

DM, dry matter; FM, fresh matter; CP, crude protein; aNDF, neutral detergent fibre with alpha-amylase; ADF, acid detergent fibre

Table 3: Effect of ensiling length on the metabolizable energy concentrations (in MJ/kg dry matter) and digestibility of organic matter (in g/100 g organic matter) of three tropical forage legumes, sorghum, and mixed sorghum-legume silages (least square means and standard error of means (SEM), n = 2))

Variables	Forages	0 d	30 d	75 d	180 d	SEM	*P*-value
	Soybean	9.0^{a}	9.0^{a}	9.1^{a}	8.5^{b}	0.09	<0.05
	Jack bean	8.6^{a}	8.1^{a}	8.1^{a}	7.2^{b}	0.11	<0.01
	Lablab	7.8^{b}	8.4^{a}	8.5^{a}	7.7^{b}	0.04	<0.01
ME	Sorghum	9.7	9.8	9.5	9.1	0.19	n.s.
	Sorghum - soybean	9.4^{ab}	9.4^{ab}	9.5^{a}	8.9^{b}	0.09	<0.05
	Sorghum - jack bean	9.3^{a}	9.0^{b}	9.0^{b}	8.4^{c}	0.04	<0.01
	Sorghum - lablab	8.9^{a}	8.9^{a}	8.9^{a}	8.5^{b}	0.07	<0.05
	Soybean	74.2^{a}	74.1^{a}	75.1^{a}	69.8^{b}	0.71	<0.05
	Jack bean	73.3^{a}	67.9^{b}	68.1^{b}	59.7^{c}	0.85	<0.01
	Lablab	66.9^{b}	71.8^{a}	71.5^{a}	65.0^{c}	0.24	<0.01
DOM	Sorghum	79.2	79.9	77.8	74.6	1.60	n.s.
	Sorghum - soybean	77.2	76.7	78.4	73.3	0.93	n.s.
	Sorghum - jack bean	76.8^{a}	74.1^{b}	74.3^{b}	68.8^{c}	0.34	<0.01
	Sorghum - lablab	74.3^{a}	74.3^{a}	74.4^{a}	70.8^{b}	0.58	<0.05

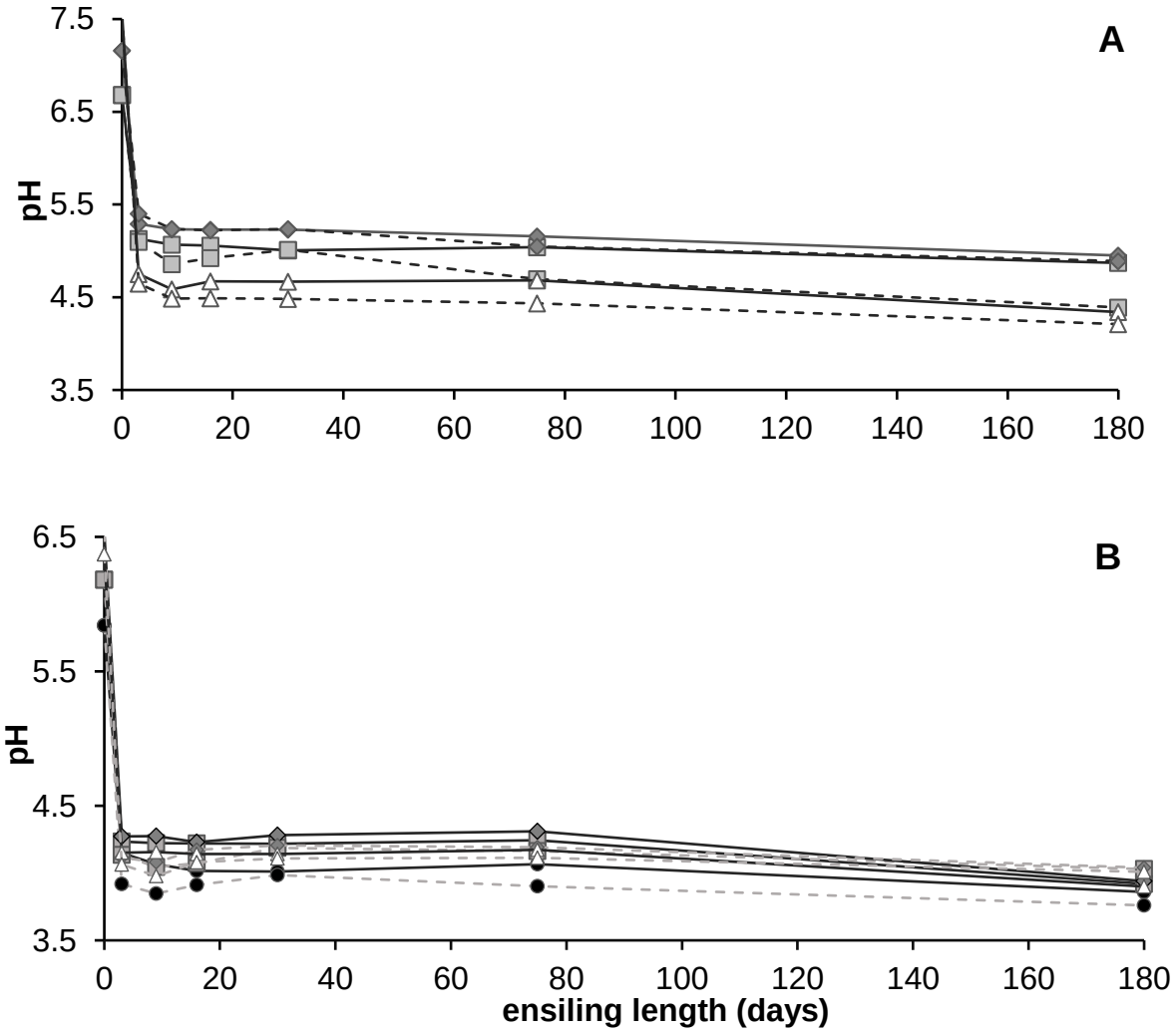
$^{a–c}$ Means within rows with different superscripts differ according to Tukey-Kramer test ($P < 0.05$)
n.s., not significant; ME, metabolizable energy; DOM, digestibility of organic matter

soybean, lablab and sorghum differs across storage temperatures. ($P < 0.01$; for all three forages).

Lactic acid concentrations of silages ranged from 11.6 to 63.1 g/kg DM with sorghum and mixed sorghum-legume silages having greater lactic acid concentrations than the sole legume silages (Table 4). An interaction effect of ensiling length and storage temperature was observed in the lactic acid concentrations of soybean ($P < 0.01$), jack bean, and sorghum ($P < 0.05$; for both forage) with a greater difference in outdoor storage than indoors at 75 d compared with a marginal difference of storage temperatures at 30 d. For lablab, lactic acid concentration decreased with ensiling length ($P < 0.01$). The concentration of lactic acid was lower in lablab silages kept outdoors than those stored indoors ($P < 0.01$), whereas a greater concentration of lactic acid was observed in sorghum-soybean and sorghum-lablab silages stored outdoors than indoors ($P < 0.01$ for all).

There was no ensiling length effect for acetic acid concentrations in soybean, jack bean, soybean-jack bean, and soybean-lablab silages, whereas the concentrations of acetic acid in lablab, sorghum and sorghum-soybean silages decreased at 75 d compared to 30 d ($P < 0.01$ for all forages and comparisons). The concentrations of acetic acid in the silages of jack bean, lablab, sorghum, and sorghum-soybean stored indoors were greater compared to those stored outdoors ($P < 0.01$).

An interaction effect between ensiling length and storage temperature was found for the lactic acid to acetic acid ratio of sorghum-soybean silage ($P < 0.05$) with the lowest lactic acid to acetic acid ratio at 30 d ensiling length and a greater absolute difference between silages stored outdoor and indoor at 75 d. There was ensiling length effect on the lactic acid to acetic acid ratio of jack bean silage ($P < 0.05$), with the lactic acid to acetic acid ratio lowered at 75 d than 30 d. The lactic acid to acetic acid ratio of sorghum silage stored indoor was greater than those stored outdoor ($P < 0.05$).



Forage	*P*-value		
	L	T	L*T
Soybean	<0.01	<0.01	<0.05
Jack bean	<0.01	n.s	n.s
Lablab	<0.01	<0.01	n.s
Sorghum	<0.01	n.s	<0.01
Sorghum – soybean	<0.01	<0.01	<0.01
Sorghum – jack bean	<0.01	<0.01	<0.01
Sorghum – lablab	<0.01	<0.05	<0.05

Figure 2: pH of silages from (A) soybean ( ), jack bean ( ), and lablab ( ) (B) sorghum ( ), sorghum-soybean ( ), sorghum-jack bean ( ), and sorghum-lablab ( ) ensiled outdoor ( ) and indoor (-----) at various ensiling lengths with statistical *P*-value (L= length; T= temperature)

Table 4: Effect of ensiling length, temperature, and their interaction on the fermentation characteristics (g/kg dry matter or as stated) of three tropical forage legumes, sorghum, and sorghum-legume silages (least square means and standard error of means (SEM), n = 3)

Variables	Forages	30 d		75 d		SEM	Length		Temperature		*P*-value		
		OD	ID	OD	ID		30 d	75 d	OD	ID	L	T	L*T
Lactic acid	Soybean	22.5	25.0	11.6	26.5	1.53	23.8	19.1	17.1	25.8	<0.01	<0.05	<0.01
	Jack bean	25.0	16.3	12.3	16.7	1.95	20.7	14.5	18.7	16.5	<0.05	n.s.	<0.05
	Lablab	39.4	52.0	16.9	41.9	3.20	45.7	29.4	28.2	47.0	<0.01	<0.01	n.s.
	Sorghum	47.9	57.4	19.2	51.8	3.92	52.7	35.5	33.6	54.6	<0.01	<0.01	<0.05
	Sorghum - soybean	50.7	38.5	49.4	38.5	0.83	44.6	44.0	50.1	38.5	n.s.	<0.01	n.s.
	Sorghum - jack bean	42.7	45.9	53.9	45.9	3.19	44.3	49.9	48.3	45.9	n.s.	n.s.	n.s.
	Sorghum - lablab	58.9	52.3	63.1	52.3	1.32	55.6	57.7	61.0	52.3	n.s.	<0.01	n.s.
Acetic acid	Soybean	8.77	9.97	7.99	8.61	1.46	9.40	8.30	8.40	9.30	n.s.	n.s.	n.s.
	Jack bean	13.2	15.3	14.2	18.3	1.33	14.3	16.3	13.7	16.8	n.s.	<0.05	n.s.
	Lablab	10.4	15.9	5.37	13.6	0.72	13.2	9.50	7.90	14.8	<0.01	<0.01	n.s.
	Sorghum	10.8	11.4	4.60	9.60	1.07	11.1	9.60	10.8	10.5	<0.01	<0.05	n.s.
	Sorghum - soybean	10.7	12.9	6.11	9.90	0.56	11.8	8.00	8.40	11.4	<0.01	<0.01	n.s.
	Sorghum - jack bean	12.2	13.3	10.2	12.7	1.43	12.8	11.5	11.2	13.0	n.s.	n.s.	n.s.
	Sorghum - lablab	12.6	11.1	11.1	12.1	1.38	11.9	11.6	11.9	11.6	n.s.	n.s.	n.s.
LA : AA	Soybean	2.62	2.53	1.70	3.27	0.40	2.60	2.50	2.20	2.90	n.s.	n.s.	n.s.
	Jack bean	1.90	1.14	0.91	0.90	0.20	1.50	0.90	1.40	1.00	<0.05	n.s.	n.s.
	Lablab	3.81	3.28	3.19	3.12	0.31	3.50	3.20	3.50	3.20	n.s.	n.s.	n.s.
	Sorghum	4.43	5.04	4.27	5.51	0.32	4.70	4.90	4.40	5.30	n.s.	<0.05	n.s.
	Sorghum - soybean	4.77	2.99	8.40	3.89	0.57	3.90	6.10	6.60	3.40	<0.01	<0.01	<0.05
	Sorghum - jack bean	3.47	3.45	5.72	3.75	0.63	3.50	4.70	4.60	3.60	n.s.	n.s.	n.s.
	Sorghum - lablab	4.66	4.94	6.07	4.37	0.64	4.80	5.20	5.40	4.70	n.s.	n.s.	n.s.
NH_3-N (g/kg N)	Soybean	21.4	21.2	33.1	37.8	3.70	21.3	35.5	27.3	29.5	<0.01	n.s.	n.s.
	Jack bean	19.6	18.3	44.7	46.5	1.90	19.0	45.6	32.2	32.4	<0.01	n.s.	n.s.
	Lablab	12.1	13.3	26.6	39.4	2.11	12.7	33.0	19.4	26.4	<0.01	<0.05	<0.05
	Sorghum	22.2	22.7	12.4	21.7	1.28	22.5	17.1	17.3	22.2	<0.01	<0.01	<0.01
	Sorghum - soybean	14.1	13.2	7.80	12.4	0.77	13.7	10.1	11.0	12.8	<0.01	<0.05	<0.01
	Sorghum - jack bean	16.5	13.2	11.5	13.8	1.56	14.9	12.7	14.0	13.5	n.s.	n.s.	n.s.
	Sorghum - lablab	13.2	12.4	12.6	16.6	1.07	12.8	14.6	12.9	14.5	n.s.	n.s.	n.s.

[a–c] Means within rows with different superscripts differ according to Tukey-Kramer test ($P < 0.05$); n.s., not significant; L, length; T, temperature; LA : AA, lactic acid to acetic acid ratio; OD, outdoor; ID, indoor

The concentration of NH_3-N ranged from 8 to 47 g/kg N, and it increased with increasing ensiling length in all sole legume silages ($P < 0.01$). An interaction effect between ensiling length and storage temperature was found for NH_3-N concentration of lablab silage ($P < 0.05$) with the lowest NH_3-N concentration at 30 d ensiling length and a greater absolute difference between silages stored outdoor and indoor at 75 d. In contrast, the lowest NH_3-N concentration with greater differentiation in the storage temperature was found at 75 d ensiling length in sorghum and sorghum-soybean ($P < 0.01$; for both forages) for the interaction effect. The NH_3-N concentrations were greater in silages of lablab, sorghum, and sorghum-soybean stored indoors compared to those kept outdoors ($P < 0.01$).

2.4.6 Fibre-bound protein characteristics

There was an interaction effect between ensiling length and storage temperature of NDIN proportion of legume silages, sorghum silage, and sorghum-jack bean silages ($P < 0.05$) with a marginal difference in silages stored at a different temperature at day 30 and a wide difference between storage temperature from 75 till 180 d during which NDIN proportion of silages stored outdoor was greater than indoor silages (Figure 3, Table 5).

For all silages, NDIN proportion was cubically affected by ensiling length, being lower after 30 d of ensiling in all silages, then increasing until 75 d and then further declining until 180 d in most silages ($P < 0.01$). The proportion of NDIN in silages stored outdoors was consistently higher than the indoors, with lablab silage having the greatest proportion of NDIN ($P < 0.01$). For ADIN proportion of lablab, sorghum and sorghum-legume silages, there was an interaction effect between the ensiling length and storage temperature (for all; $P < 0.01$) with lesser differences in ADIN proportion between indoor and outdoor storage at day 30 and 180 but greater differences at day 75. A cubic response to ensiling length was found for ADIN proportion of lablab, jack bean and sorghum-lablab silage ($P < 0.01$) in a similar manner to that of NDIN proportion, whereas a quadratic effect of ensiling length was found for ADIN proportion of sorghum-soybean and sorghum-jack bean silage ($P < 0.01$). There was an effect of storage temperature on ADIN proportion of lablab, sorghum and sorghum-legume silages ($P < 0.01$). An interaction effect between ensiling length and storage temperature was found for the proportion of B_3 of lablab ($P < 0.01$) and sorghum-

lablab ($P < 0.05$). For lablab, a major difference was observed in the B_3 proportion between the storage temperature after day 30 till 180, during which the proportion of B_3 was higher in silages stored outdoor than those stored indoors. The interaction effect in sorghum-lablab showed a higher proportion of B_3 in silages stored outdoor than indoor till day 75, after which the proportion of B_3 was reduced in silages stored outdoor compared to indoor. There was a cubic effect on ensiling length for the proportion of B_3 in all silages (for all; $P < 0.01$). The proportion of B_3 was higher in silages of jack bean and lablab stored outdoor compared to indoor ($P < 0.01$). In contrast, there was no difference in the proportion of B_3 in the remaining silages as affected by storage temperature.

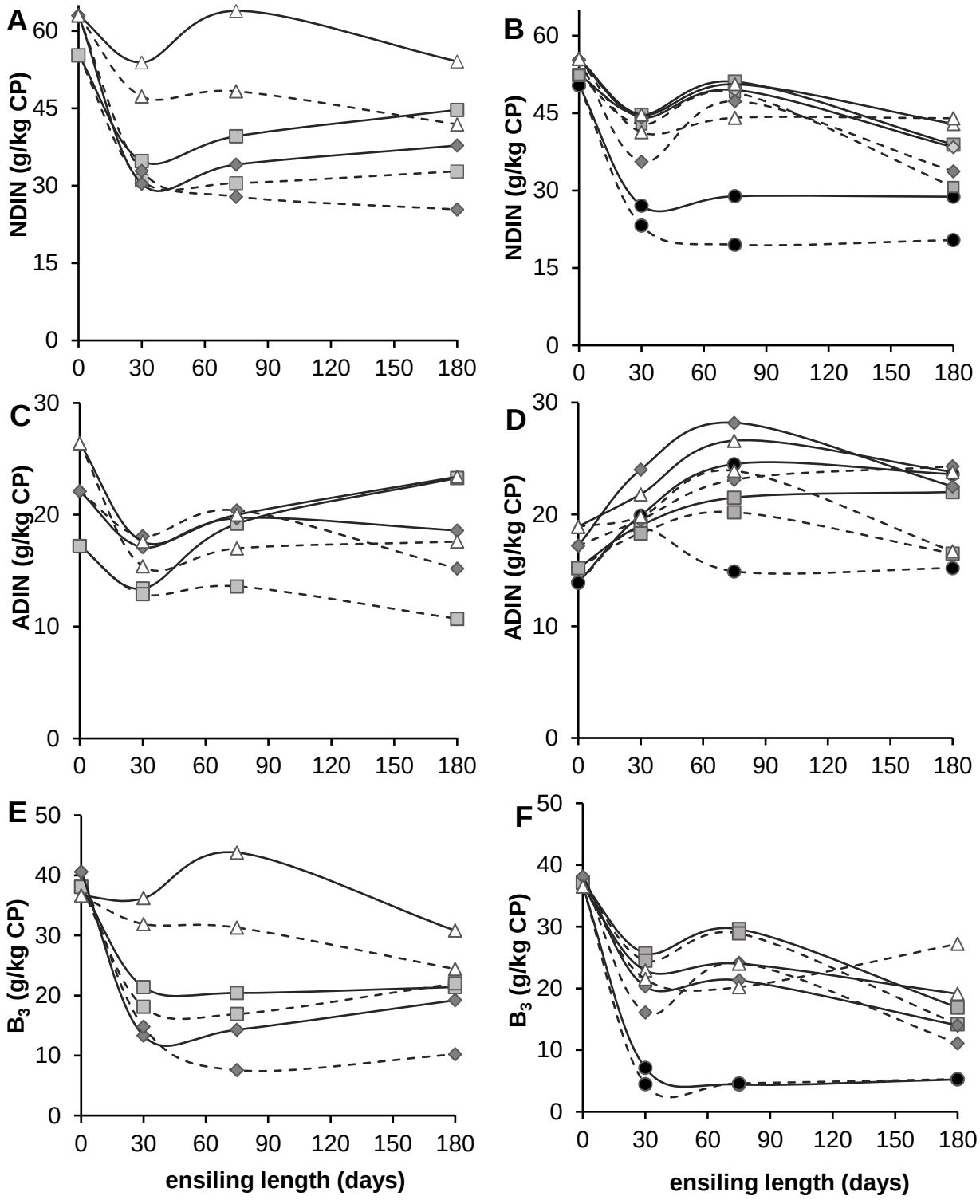


Figure 3: The proportions of NDIN, ADIN and B_3 (g/kg CP) of soybean (□), jack bean (◇), lablab (△) ensiled alone (Panel A, C, E) or their mixtures with sorghum as well as sorghum alone (●) (Panel B, D, F)ensiled under outdoor (——) or indoor (-----) conditions at various conservation lengths.

Table 5: Statistical *P*-values and contrasts of the effect of ensiling length, temperature and their interaction on the fibre bound protein characteristics (g/kg CP) of three tropical forage legumes, sorghum and mixed silages shown in Figure 3

Variables	Forages	*P*-value			Contrasts		
		L	T	L*T	Lin	Quad	Cub
	Soybean	<0.01	<0.01	<0.05	<0.01	<0.01	<0.01
	Jack bean	<0.01	<0.05	<0.05	<0.01	<0.01	<0.01
	Lablab	<0.01	<0.01	<0.01	<0.01	0.02	<0.01
NDIN	Sorghum	<0.01	<0.01	<0.05	<0.01	<0.01	<0.01
	Sorghum – soybean	<0.01	<0.05	n.s.	<0.01	n.s.	<0.01
	Sorghum – jack bean	<0.01	<0.01	<0.05	<0.01	n.s.	<0.01
	Sorghum – lablab	<0.01	n.s.	n.s.	<0.01	<0.01	<0.01
	Soybean	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Jack bean	<0.01	n.s.	n.s.	<0.01	n.s.	<0.01
	Lablab	<0.01	n.s.	n.s.	<0.01	n.s.	<0.01
ADIN	Sorghum	<0.01	<0.01	<0.01	<0.01	<0.01	<0.05
	Sorghum – soybean	<0.01	<0.01	<0.01	<0.01	<0.01	n.s.
	Sorghum – jack bean	<0.01	<0.01	<0.01	<0.01	<0.01	n.s.
	Sorghum – lablab	<0.01	<0.01	<0.01	n.s.	<0.01	0.01
	Soybean	<0.01	n.s.	n.s.	0.01	<0.01	0.03
	Jack bean	<0.01	<0.05	n.s.	<0.01	<0.01	<0.01
	Lablab	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
B_3	Sorghum	<0.01	n.s.	n.s.	<0.01	<0.01	<0.01
	Sorghum – soybean	<0.01	n.s.	n.s.	<0.01	n.s.	<0.01
	Sorghum – jack bean	<0.01	n.s.	n.s.	<0.01	<0.01	<0.01
	Sorghum – lablab	<0.01	n.s.	<0.05	<0.01	<0.01	<0.01

n.s., not significant; L, length; T, temperature; Lin, linear; Quad, quadratic; Cub, cubic; NDIN, neutral detergent insoluble nitrogen; ADIN, acid detergent insoluble nitrogen; and B_3, slowly degraded crude protein fraction

2.5 Discussions

2.5.1 Nutrient concentrations

Concentrations of all nutrient fractions differed between forages before and after ensiling in the present study; yet, the extent of changes in the nutrient concentrations depended on the duration of storage as expected. The DM concentration of all silages was greatest at 75 d of storage before it declined again. The rise in the DM concentration during the first 75 d of ensiling is probably related to the rapid acidification due to the added LAB inoculant that supplies the lactic acid required for fermentation activity until stable fermentation was achieved. According to Muck *et al.* (2018), DM recovery in silages treated with LAB inoculants compared to untreated silages is higher because of a rapid reduction in silage pH and higher lactic acid concentration. However, the continued microbial respiration and fermentation process, though at a reduced rate, contributes to the loss of soluble nutrients, particularly the hemicellulose fraction. These losses may be the cause of the decline observed in DM concentrations beyond 75 d of storage. Contrasting results have been found related to the changes in the DM concentration of silages during ensiling. Yahaya *et al.* (2001) found a decrease in the DM concentration of alfalfa silage as ensiling length progressed (56 d), whereas storage length did not influence the DM concentration of corn silage in the studies of Ferraretto *et al.* (2015; 240 d) and Gerlach *et al.* (2018; 120 d). Moreover, Santos and Kung (2016) showed that the DM concentration of alfalfa silage with high DM concentrations (450 g/kg DM) increased considerably with advancing duration of storage (360 d) as compared to alfalfa silage with low DM concentration (330 g/kg DM) that was relatively constant with the duration of storage. Thus, the study of Santos and Kung (2016) found that greater losses at lower forage DM concentrations were associated with the fibre fraction; nevertheless, in the present study, the effects of ensiling length on DM concentrations were similar for all silages despite varying in original DM concentration. Hence, the reduced hemicellulose fraction in all silages in the present study until 75 d except in jack bean silage, where CP concentration also reduces, could be responsible for the increased DM concentration until that length of storage.

Concentrations of CP differed in forages before and after ensiling and increased linearly with ensiling length till day 75 before decreasing significantly at day 180 in most silages.

The greater CP concentration of most silages at 75 d than 180 d could be related to the LAB inoculant preserving the CP by supplying additional substrate to increase pH reduction (Polan *et al.*, 1998). Additionally, the decrease in the hemicellulose fraction of most silages could be responsible for the rise in CP concentration because the CP's trends are similar to the DM for most silages. Deviating from this trend was jack bean silage CP concentration that reduces all through with ensiling length and sorghum silage with no effect of ensiling length. A probable cause for the linear decline in CP concentrations in jack bean silage from 0 to 180 d may be due to its high CP solubility and high buffering capacity (Heinritz *et al.*, 2012), which promote proteolytic activity during ensiling (Van Soest, 2018). This explanation is supported by the high pH and NH_3-N concentration compared to other silages after 75 d of ensiling.

The lower CP concentration of outdoor silages compared with indoor silages is likely a result of increased proteolysis rates, which increase exponentially with increasing storage temperatures between 10 and 40°C (McKersie, 1981) and thus outdoor temperatures (Muck and Dickerson, 1988). Silages stored outdoors in the present study spent considerable hours under temperature that could enhance proteolysis rate.

Changes in the aNDF and ADF concentrations as ensiling length advances differ among silages and may depend on the ensiled forage's physicochemical properties. The continued hydrolytic activity, either from forage enzymatic activity or due to the silo acidic conditions, have been suggested for changes in the structural carbohydrates of forages after long-term ensiling (Rooke and Hatfield, 2003). Indeed, previous studies have shown an increase, decrease, or even no changes in aNDF and ADF concentrations of corn and alfalfa silages with a prolonged ensiling length of about 365 d (Der Bedrosian *et al.*, 2012; Gerlach *et al.*, 2018; Santos and Kung, 2016). The continuous increase in aNDF and ADF concentration of jack bean silage as the ensiling progress suggests higher losses of soluble nutrients as seen in CP concentration losses. *Lactobacillus plantarum,* as contained in the added LAB inoculant, has the potential of utilizing the structural carbohydrate pool for fermentation (Rooke and Hatfield, 2003). Thus, the reduced concentrations of the hemicellulose fraction observed in most silages at 75 d of storage compared to the forage before ensiling is probably due to the continued hydrolytic activity from forage enzymatic activity or its use as a substrate for

LAB growth. In contrast, the greater aNDF and ADF concentration at 180 d than other days is possibly related to the losses of DM concentration (CP constituents) at this length of storage.

Temperature, pH and time have been reported to affect the hydrolytic activity of forage enzymes (McDonald *et al.*, 1991). Hence, the increase in the aNDF and ADF concentration of silages stored outdoors in the present study is probably also related to the deactivation of enzymes that hydrolyze structural carbohydrates at outdoor storage temperatures. Besides, the increased CP degradation, as shown by the greater concentration of NH_3-N outdoor than indoor silages, could also be responsible for the rise in the aNDF and ADF concentration.

The decrease in the concentrations of ME and DOM of most silages with advancing ensiling length in the present study suggests that most of the fermentable substrates required for rumen microbes for ME production and digestibility purposes have been used by silage microbes during ensiling. Since digestibility depends on both cell wall concentration and its availability for digestion (Van Soest, 2018), the higher aNDF and ADF concentrations found in silages with the increase in ensiling length may be reflect a loss of cell contents like soluble carbohydrates, thus leading to the decline in the concentrations of ME and DOM.

2.5.2 Silage fermentation characteristics

Fermentation parameters as affected by the ensiling length and storage temperature were determined until 75 d. The pH and concentrations of acetic and lactic acids of all silages were lower at 75 than at 30 d of ensiling, whereas NH_3-N proportions were greater in all legume silages at 75 d.

Silage pH remains the traditional indicator of satisfactory fermentation, and achieving a pH ≤ 4 in silage making is a conventional approach to assess successful preservation (Kaiser *et al.*, 2004). The reduced pH with increasing ensiling length indicates that silage microbes acidic end products reduce silage pH in favour of acid-tolerant lactic acid bacteria (LAB) growth. In general, the final pH at 180 d in legume silages (4.60) was greater than the sorghum (3.81) and sorghum-legume (3.97) silages, and the buffering capacity of forage depends on the concentrations of organic acids and their salts and CP contributions of about 10 - 20% (Kaiser *et al.*, 2004). Thus, the low water-soluble carbohydrate (WSC) concentration

and the high CP concentration accustomed to legume silages may be responsible for their high pH, whereas the low pH in the sorghum-legume silages is a reflection of a greater concentration of lactic acid as observed in the present study which is in agreement with previous studies (Bakken *et al.*, 2017; Hartinger *et al.*, 2019; Krawutschke *et al.*, 2013).

It was anticipated that temperature would influence the fermentation process because ensiling involve microbiological and enzymatic activities that are greatly affected by their environment, such as moisture and temperature (Muck *et al.*, 2003). The average final pH of all silages stored outdoor (4.30) was slightly greater than those kept indoors (4.19). In the plastic totes stored outdoors, temperatures exceeded 40°C for at least 7 h daily, which may likely inhibit microbial activity in the silages because most of the LAB responsible for the decline in silage pH can grow optimally at ambient temperatures between 25°C and 40°C (Rooke and Hatfield, 2003).

Typically, the fermentable substrates in grasses and cereal crops are greater than forage legumes, and this is vital for lactic acid production during silage fermentation (Heinritz *et al.*, 2012). As observed in the present study, the lactic acid concentration of legume and sorghum silages was about 35% lower after 75 d than 30 d of ensiling, whereas a slight increase in lactic acid concentrations was observed for sorghum-legume silages. The lactic acid reduction at 75 d suggests a limited concentration of fermentable substrates, thereby inhibiting LAB growth after stable fermentation has been achieved; besides, the presence of lactic acid degrading microbes like fungi and enterobacteria could cause the reduction (Rooke, 1991). On the other hand, the fermentable substrates in sorghum-legume silages may be higher to have enhanced the lactic acid concentration at 75 d of ensiling.

The lactic acid concentration was less in sole legume and sorghum silages at temperatures outdoors in the present study. The lower concentration of fermentable substrates at ensiling and the restriction in LAB growth at high temperatures might have further lowered the lactic acid concentration of sole silages at temperatures outdoors. This finding agrees with the studies of Weinberg *et al.* (2001) and Kim and Adesogan (2006) that showed reduced lactic acid concentration in wheat and corn silages at ensiling storage temperatures of 41 vs 28°C as this exceeds the optima temperature for LAB growth. Nevertheless, sorghum-legume silages stored outdoors had greater lactic acid concentrations than those kept

indoors, probably because of the extensive hydrolysis of structural carbohydrates at high temperatures, which releases sugars for fermentation (Muck *et al.*, 2003). The effect of temperature in a true sense cannot be exclusive, but in a situation where there are enough fermentable substrates, there may be a favourable shift, as found in the sorghum-legume silages.

The acetic acid concentrations were lower in all silages at 75 d than 30 d of ensiling, except in jack bean silages, in which acetic acid concentrations were even 14% greater at 75 d. The addition of homofermentative LAB inoculant before ensiling may be responsible for the lower production of acetic acid recorded in most silages because it is more favourable for lactic acid production. However, under sugar-limiting conditions, like when ensiling legumes, *Lactobacillus plantarum* could degrade lactic acid anaerobically to form acetic acid after prolonged storage (Lindgren *et al.*, 1990; Rooke, 1991). This shift from lactic to acetic acid production may be responsible for the greater acetic acid concentration in jack bean silage at 75 d than 30 d of ensiling. Additionally, as ensiling progressed, the lactate to acetate ratio of jack bean, among other legume silage, declined below 1, an indicator of an abnormal fermentation (Kung *et al.*, 2018). This result may indicate that alongside the LAB inoculant, the greater concentrations of organic acids in jack bean than the other forages could have facilitated the shift from lactic to acetic acid production during fermentation. Nevertheless, the abundance of fermentable substrates in the sorghum-legume silages increased the lactate to acetate ratio, which was beyond the critical value of 2 (Kaiser *et al.*, 2004).

Ammonia-nitrogen in silages is primarily an end product of proteolysis by plant and microbial enzymes (Rooke and Hatfield, 2003). The present study shows that NH_3-N concentrations were greater at 75 d than 30 d of ensiling for all legume silages, whereas those in sorghum or the sorghum-legume silages decreased. Characteristically, the concentration of soluble CP is greater in legumes than in grasses, which results in high NH_3-N concentrations in legume silages (Wang *et al.*, 2009). Additionally, the high NH_3-N concentration could also suggest high enterobacteria or clostridial activity; however, butyric acid concentration was not measured in the present study to substantiate this assumption.

High-temperature conditions (10°C - 40°C) increase the activities of plant proteases, causing extensive hydrolysis of protein (Muck and Dickerson, 1988) to amino acids and further degradation to NH_3-N by microbes. In the present study, the effect of storage temperature on NH_3-N concentration was minor in most silages except in lablab, sorghum, and sorghum-soybean silages stored indoors with greater NH_3-N concentration. It appears that the warm conditions in the outdoor silages were not enough to result in greater concentrations of NH_3-N which may suggest inhibition of proteolytic microbes or stimulation of protein condensation with fibre (Van Soest, 2018) as found in the greater proportion of fibre-bound protein of outdoor silages compared to indoor silages in the present study.

2.5.3 Fibre-bound protein characteristics

The proportion of CP fractions NDIN, ADIN, and B_3 was lower in most silages in comparison to the original forage ensiled, except the proportion of ADIN in sorghum and sorghum-legume silages, which was greater in the silages than the fresh forages. Besides, there was an interaction between ensiling length and temperature for either NDIN or ADIN proportion in all silages. The response of the fibre bound protein proportion to prolonged storage varies largely among the sole silage and between the sole and sorghum-legume silages. This variation could be attributed to the difference in protein solubility, soluble carbohydrate and fibre matrix of the ensiled forage (Van Soest, 2018).

Reduction in the NDIN proportion of drier legume silages was rapid in the first 30 d than sorghum-legume silages with high WSC presumably. There seems to be a rapid degradation of the hemicellulose fraction during the first 30 d of ensiling to achieve stable fermentation, after which the cellulose fraction increased slightly, resulting in a higher NDIN proportion at day 75 than day 30. The continuation of proteolysis or unavailable WSC after day 75 could be responsible for the reduction in NDIN proportion at 180 d of ensiling in legume silages.

The increase in ADIN proportion depends largely on the decrease in protein solubility and the proportion of reactive carbohydrates (hemicellulose and soluble carbohydrates) available for Maillard reaction (Van Soest, 2018). As seen in the present study, the steady rise in ADIN proportion of sorghum and sorghum-legume silages from 0 to 75 d in

comparison to the legume silages with reduced and increased ADIN proportion at 30 d and 75 d may be due to more reactive hemicellulose that was hydrolyzed and moisture that is available for condensation with amino groups during the Maillard reaction. Although the DM concentration of all silages increased from 0 to 75 d, the moisture concentration in sorghum and sorghum-legume silages was greater than legume silages. It appears that moisture concentration plays a more significant role in ADIN formation. In contrast, the declension of ADIN proportion after 75 d of ensiling further establish the continuation of protein degradation or the absence of reactive carbohydrates.

Extended heating from microbial respiration leads to the polymerization of amino acids with carbohydrates (van Soest and Mason, 1991). These processes raised the silo temperature, thereby increasing the Maillard reaction rate, which became clear by the increment of NDIN and ADIN in the silages when stored outdoors than indoors. The initial decrease but later increase in NDIN proportion of outdoor silages could be related to the losses associated with extensive hydrolysis of structural carbohydrates before fermentation was stabilized and not without protein degradation. Moreover, an interaction with ensiling length was observed, in which NDIN proportion of some of the legume silages stored outdoors kept increasing until 180 d, while silages stored indoors appeared to stabilize after 75 d. Interestingly, for the sorghum-legume silages, a clear cubic relationship was observed where the drop in NDIN was beyond the original concentration of the forages. The proportion of ADIN behaved differently, clearly increasing with ensiling length, with a maximum at 75 d. This discovery corroborates the previous study that shows that the increase in Maillard reaction is not only a function of high temperature but also of the length of exposure (Muck *et al.*, 2003).

The proportion of the slowly degraded protein fraction (B_3), which is a function of protein solubility, declines as the duration of storage increases in all silages. This reduction seems to be mediated by a rise in ADIN over time, thereby reducing the proportion of extensins (cell wall-bound protein). The effect of storage temperature on the proportion of B_3 of jack bean and lablab compared to other unaffected could be related to the ADIN proportion elevated by high temperature.

Based on this study's findings, ensiling length clearly affects nutrient concentration, fermentation quality, and fibre-bound protein characteristics of all silage with the same observable pattern in both sole and sorghum-legume silages in response to ensiling length. Although the same trend was observed, a large variation exists among legume silages compared to mixed silages, which appears to depend on the difference in the physicochemical properties among forages at ensiling. This difference seems to dictate the mechanism of action of the added LAB inoculant and the rate at which substrates were provided for fermentation processes. Remarkably, the proportion of soluble nutrients preserved in most silage until 75 d of ensiling declined considerably, thereby increasing DM and CP losses, fibre concentration and reducing digestibility afterwards. Besides, temperature affected the fermentation and fibre-bound protein characteristics with higher variation in the fibre bound protein of legume silages than sorghum and the sorghum-legume silages. The interaction of ensiling length and temperature as observed in the fibre-bound protein further validates that increase in Maillard reaction is not only a function of high temperature but also of the length of exposure (Muck *et al.*, 2003). Overall, the response of forages to ensiling length is very dynamic, and a sort of stabilization seems to occur after some time. Short periods of ensiling can have different characteristics than long periods of ensiling. The qualities of the sorghum-legume silages were better than the legumes in response to the factors studied with lablab, and its mixture appeared to be more desirable while jack bean behaved oppositely. In summary, the ensiling of legumes and their mixtures over a prolonged period in a high ambient temperature typical of tropical regions raises silo temperature, negatively affecting fermentation and nutritional quality.

2.6 Conclusions

This study demonstrates the dynamism in the response of ensiled forages and their mixtures to ensiling length. It becomes clear that a short period of ensiling preserves the nutritional quality of ensiled forages compared to prolonged storage that increases nutrient losses. The storage temperature mainly affected the fibre-bound protein characteristic over time, especially in the sorghum-legume silages. Moreover, legume silages stored outdoor appears unstable. This study further shows the ensilability of tropical legumes and the

improvement in legumes' silage quality when mixed with sorghum. Lablab and sorghum-lablab mixture silage quality appears to be the best in response to the factors studied compared to other silages. Provision of shade under conditions of intensive sunlight or high temperatures are recommended to prevent poor silage quality. Additionally, proper changes in ration formulation should be implemented to verify the transformation in the nutritional value of the forage that happened during prolonged storage.

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Chapter 3

3. *In vitro* rumen fermentation and post-ruminal digestibility of sorghum-soybean forage as affected by ensiling length, storage temperature and its interactions with crude protein levels

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3.1 Abstract

The study aimed to evaluate the effects of ensiling length, storage temperature, and its interaction with crude protein (CP) levels in sorghum–soybean forage mixtures on in vitro rumen fermentation and post-ruminal digestibility of nutrients. The dietary treatments consisted of fresh forages (d 0) and silages of sorghum and soybean stored indoors or outdoors for 75 and 180 d with additional ingredients to make two dietary CP levels, 90 and 130 g/kg dry matter (DM) and a forage-to-concentrate ratio of 80 to 20. An in vitro procedure was conducted using the ANKOM RF technique to study rumen fermentation. The dietary treatments were incubated in duplicate for 8 and 24 h in three runs. After each incubation time, in vitro rumen fermentation parameters were measured, and the protozoa population was counted using a microscope. Post-ruminal digestibility was determined using the pepsin and pancreatic solubility procedure. Cumulative gas production (GP) increased quadratically with ensiling length (8 h, $p < 0.01$; 24 h, $p = 0.02$), and the GP differed between CP levels at both incubation times ($p < 0.01$). However, total short-chain fatty acid (SCFA) concentrations in rumen inoculum increased quadratically with ensiling length ($p < 0.01$; for both incubation times), and interaction between ensiling length and CP levels was observed in proportions of acetate and propionate after 24 h of incubation ($p < 0.01$; for both incubation times). Similarly, an interaction between ensiling length and CP levels was found in the proportion of valerate after 24 h of incubation ($p < 0.01$). There was a quadratic response to ensiling length in the NH_4–N concentration after 8 h ($p < 0.01$) and 24 h ($p < 0.05$), and the CP level also differed ($p < 0.01$) at both incubation times. The ciliate protozoa count after 24 h was higher in low CP diets than in high CP diets ($p = 0.04$). The amount of CP in the undegraded substrate at both incubation times differed between CP levels ($p < 0.01$; for both incubation times). An interaction effect between ensiling length and storage temperature after 8 h ($p = 0.02$) and 24 h ($p < 0.01$) was observed for intestinal CP digestibility. The effect of CP levels on intestinal CP digestibility differed after 8 h ($p < 0.01$) and 24 h ($p < 0.01$). In conclusion, increasing ensiling length beyond 75 d reduced CP digestibility, and additional CP inclusion did not ameliorate this.

Keywords: ensiling; temperature; crude protein; rumen fermentation; intestinal digestibility

3.2 Introduction

The microbial degradation of carbohydrates and crude protein (CP) in the rumen by diverse microbes with an expected net feeding strategy is a complex process that takes place before nutrient absorption post-ruminally. To assess the nutritional value of dietary CP in ruminants, the amount of undegraded CP in the rumen that flows to the duodenum, its intestinal digestibility, and the amino acid composition of the undegraded CP is required (Tamminga et al. 1994; Vérité et al. 1987). The flow of rumen undegraded CP post-ruminally may vary among forage species and between fresh forages and silages (González et al. 2007). Moreover, a greater flow of undegraded CP does not always lead to increased amino acid absorption (Bach, Calsamiglia, and Stern 2005). For instance, Cone et al. (2006) reported an increased rumen undegraded CP concentration and a reduced intestinal digestibility of rumen undegraded CP for *Lolium perenne* grass. In contrast, a decrease in rumen undegraded CP concentration and increased intestinal digestibility of rumen undegraded CP was found for *Lolium perenne* grass silage using *in situ* and *in vitro* methods. In another study by Lima and colleagues (Lima et al. 2011), the digestible CP supply in the small intestine of sheep fed fresh sorghum–soybean was lower than the intestinal digestible CP supply for ensiled sorghum-soybean forage.

The differences in the growth habits and morphological characteristics of diverse tropical forage legumes showed differences in the CP digestibility of tropical forage legumes in ruminants (Castro-Montoya and Dickhoefer 2018). Moreover, the protein quality and the CP fractions of tropical forage legumes that differs between species changed during conservation techniques (Aloba et al., 2022; Castro-Montoya and Dickhoefer 2020). In view of the impacts of ensiling conditions, such as a prolonged ensiling period and storage temperature, the variation in the intestinal CP digestibility between fresh and silage of forage mixtures may be amplified. For instance, ensiling three different tropical forage legumes and their combinations with sorghum over a prolonged period of 180 d in a high ambient temperature increased the proportion of acid detergent insoluble nitrogen (ADIN), decreased the neutral detergent insoluble nitrogen (NDIN), and the slowly degraded CP fraction (Aloba et al. 2022). In consideration of the preceding, changes in the CP of ensiled forages are imminent, and the CP supply from ensiled legumes for rumen microbes and post-ruminal use may be limiting. Diets containing tropical forage legume silage resulted in higher performance (average daily

gain, milk yield) than those without tropical legume silage inclusion, and it is assumed that forage legume CP appeared to be more digestible post-ruminally (Castro-Montoya and Dickhoefer 2020). Thus, evaluating how diets with ensiled forage legumes behave under low and high (relatively moderate) CP conditions with a premise that if CP from ensiled forage legumes is limiting, then under low CP conditions, negative effects would be exacerbated. Similarly relevant is to know how ensiling conditions affect the contribution of CP post-ruminally since it is generally considered that silages CP are extensively degraded in the rumen (Givens and Rulquin 2004), and that the intestinal digestibility of undegraded CP cannot be assumed to be constant for a particular feed (Hvelplund and Weisbjerg 2000). Thus, the present study evaluated the *in vitro* rumen fermentation and post-ruminal digestibility of diets containing fresh or ensiled sorghum and soybean forage combination as affected by the ensiling length, storage temperature, and its interaction with dietary CP levels.

3.3 Materials and methods

3.3.1 Experimental diets

In the present *in vitro* study, three factors were studied: the effect of ensiling length (i.e. 0, 75 and 180 days), the impact of storage temperature (i.e. indoor vs outdoor) and the effect of dietary crude protein levels (high vs low). Sorghum and soybean forage samples ensiled under different storage temperatures and lengths from a previous silage experiment (Aloba et al. 2022) were used as basal feeds mixed with other ingredients to form the diet. Details on the agronomic practices, ensiling conditions, and the chemical and fermentation characteristics of the forage samples used in the current study have been described in a previous silage experiment. The average storage temperature observed over the ensiling period for silages stored indoors and outdoors are 25 and 30°C respectively. Diets were formulated at a constant forage-to-concentrate ratio of 80 to 20 (on a dry matter (DM) basis), with sorghum forage or silage representing 48 % and soybean forage or silage 32 % of the diets (on a DM basis). The concentrate mixture in the diets comprised corn starch (9444.1, Roth GmbH, Karlsruhe, Germany) and soy protein (066-974, ProFam® 974 ADM, Illinois, USA) in different proportions to achieve diets with two CP concentrations (low CP; 90 g/kg DM and high CP; 130 g/kg DM). In addition to the fresh forage diet at 2 levels of CP concentration, 8

dietary treatments were tested (2 storage temperature × 2 ensiling lengths × 2 dietary CP concentrations).

3.3.2 In vitro fermentation with ANKOM RF technique

The in vitro experiment was conducted using an ANKOM RF gas production system (ANKOM Technology, Macedon, NY, USA) equipped with 22 units, which releases the accumulated gas automatically in the flask headspace at 0.7 psi pressure through an ANKOM sensor module.

Before the morning feeding, rumen fluid (2.9 L/incubation) was collected from various locations within the rumen of three rumen-cannulated dry Jersey cows using a perforated hose attached to a vacuum pump. All cows had free access to fresh drinking water and were fed ad libitum a total mixed ration composed of (per kg DM) corn silage (329 g), grass silage (329 g), grass hay (229 g), barley straw (100 g), urea (5 g), and a mineral mixture(8 g: 0.4 g calcium, 1.3 g phosphorus, 1.4 g magnesium, and 4.9 g sodium).

Rumen fluid was transported to the lab in a pre-warmed, insulated flask and strained through a gauze bag of 100-µm-pore size. The strained rumen fluid was mixed with a preheated (39 °C) standard buffer solution according to Menke and Steingass (1988) under constant stirring and continuous flushing with carbon dioxide in a water bath (39 °C).

Each substrate ingredient (480 mg of sorghum, 320 mg of soybean, and 200 mg of corn starch or soy protein + corn starch) was weighed separately into 500 mL Duran bottles for every run

Table 1. Chemical composition of forages as affected by ensiling length and storage temperature

Forage	Variable	Ensiling lengths			Storage temperature	
		0 d	75 d	180 d	25°C	30°C
Sorghum	Dry matter	925	890	902	893	900
	Organic matter	911	913	916	916	913
	Crude protein	99	96	96	94	99
	Neutral detergent fibre	506	418	496	465	451
	Acid detergent fibre	255	241	290	198	187
	ADFom (g/kg OM)	250	252	281	255	278
	Crude lipid	25	29	22	25	25
	ADIN	1.37	1.85	1.87	2.45	0.98
	Metabolizable energy (MJ/kg DM)	9.7	9.5	9.1	9.4	9.1
Soybean	Dry matter	924	924	925	920	928
	Organic matter	914	909	906	904	912
	Crude protein	148	170	155	160	166
	Neutral detergent fibre	412	416	507	485	439
	Acid detergent fibre	297	296	376	354	318
	ADFom (g/kg OM)	274	291	332	308	316
	Crude lipid	14	21	19	19	21
	ADIN	2.54	2.79	2.47	3.24	2.02
	Metabolizable energy (MJ/kg DM)	9.0	9.1	8.5	8.9	8.6

ADIN, Acid detergent insoluble nitrogen; ADFom, Acid detergent fibre after ashing

Table 2. Ingredient and chemical composition of the experimental diets at different crude protein levels for the *in vitro* fermentation

Ensiling length	0 d		75 d				180 d			
Storage temperature			25°C		30°C		25°C		30°C	
Crude protein levels (g/kg DM)	90	130	90	130	90	130	90	130	90	130
Ingredient composition of diets (g/kg as fed basis)										
Sorghum	480	480	480	480	480	480	480	480	480	480
Soybean	320	320	320	320	320	320	320	320	320	320
Soy protein[1]	0	100	0	100	0	100	0	100	0	100
Corn starch[2]	200	100	200	100	200	100	200	100	200	100
Chemical composition of the diets (g/kg DM)										
Organic matter	929	921	926	919	931	924	932	925	927	920
Crude protein	92	132	96	136	92	132	92	132	88	129
Neutral detergent fibre	375	408	332	365	336	369	381	414	420	453
Acid detergent fibre	217	227	211	220	210	219	246	255	273	282
Crude lipid	16.5	17.0	20.5	21.0	20.5	21.0	17.2	17.7	16.1	16.6
Non-structural carbohydrates	446	364	478	397	483	402	442	361	403	321
ADIN	1.47	2.91	1.44	2.88	2.11	3.55	1.26	2.70	2.12	3.56
Metabolizable energy (MJ/kg DM)	9.53	9.65	9.55	9.68	9.38	9.50	9.15	9.27	9.00	9.12

DM, dry matter; ADIN, acid-detergent-insoluble nitrogen; soy protein (crude protein 434 g/kg DM, crude lipids 5.0 g/kg DM, crude ash 75.3 g/kg DM); corn starch (crude protein 2.0 g/kg DM, crude ash 1 g/kg DM

to compose a total of 2 g of mixed substrate. Subsequently, 300 mL of rumen inoculum were added to each Duran bottle, its headspace saturated with carbon dioxide,sealed, and placed in a water bath at 39 ˚C for 8 h and 24 h incubation periods. Within each run, each experimental diet was incubated in duplicate per incubation period. Additionally, two blank bottles per incubation time with only rumen inoculum were included in each run to correct gas production (GP), total short-chain fatty acid (SCFA) concentration, and apparent degradability.

3.3.3 Sampling

At the end of each incubation period GP measurement was recorded. Additionally, the incubation medium's pH was recorded using a pH-meter (WTW Multi 340i, WTW, Weilheim, Germany). Then, an aliquot of 750 µl of incubation medium was taken for protozoa count. The aliquot was fixated with 750 µl of methyl green formalin-saline solution (10 mL formaldehyde solution (35%, v/v); 90 mL distilled water; 0.06 g methyl green; 0.8 g sodium chloride) and stored at 4°C in a refrigerator until counting.

Afterwards, the remaining contents of each Duran bottle were transferred to polyethene bottles and centrifuged at 500 × *g* at 4°C for 10 min (Hettich Rotanta, Tuttlingen, Germany). Two aliquots of 5 mL of decanted supernatant were collected and stored at -20 °C for determination of SCFA and ammonium-nitrogen concentrations. After centrifugation and decantation of the supernatant, the residual pellet (*in vitro* apparently degraded DM) was obtained, lyophilized, weighed, and ground it using a ball mill (Retsch, MM200, Haan, Germany) for 2 min at a frequency of 30 s, and then stored at room temperature until the determination of *in vitro* intestinal digestibility.

3.3.4 Chemical analysis

The DM, crude ash, and ether extract concentrations of each ingredient were deter- mined according to the official analytical method in Germany (VDLUFA 2012) in duplicates. Nitrogen (N) was analyzed by Dumas combustion using a Vario MAX CN element analyzer (Elementar Analysensyteme GmbH, Hanau, Germany) to determine crude protein (CP) concentrations (CP = N × 6.25) concentration (method 4.1.1 of VDLUFA). Similarly, N concentrations in residual pellets were also determined using a CN analyzer. The concentrations of neutral detergent fibre (aNDF; assayed with heat-stable amylase and sodium sulphite) and acid detergent fibre (ADF) were analyzed in sequence using the

ANKOM 200 fibre analyzer (ANKOM Technology, New York, USA) (methods 6.5.1 and 6.5.2 of VDLUFA). Each substrate's nutrient concentrations were then calculated from the chemical composition of each ingredient in duplicates (Tables 1 and 2).

For SCFA analysis, 2 mL of each aliquot of the supernatant obtained from initial centrifugation was transferred into vials and later centrifuged at 20,000 × *g* at 4°C for 10 min (Avanti™ 30, Beckman Coulter™, Indianapolis, IN, USA). An aliquot of 720 µl of the supernatant of this centrifugation was pipetted into a 1.5-mL-vial, mixed with 80 µl of an internal standard (1 mL methyl valeric acid dissolved in 99 mL formic acid), and stored at 4°C to precipitate the soluble proteins (Castro-Montoya et al., 2011). Following this, the mixture was centrifuged at 20,000 × *g* (10 min, 4°C), and 800 µl of the supernatant was transferred into 1.5-mL glass vials before analyzing for SCFA by a gas chromatograph (GC 14-A Shimadzu Corp., Kyoto, Japan) equipped with an auto-injector (AOC–20i, Shimadzu Corp., Kyoto, Japan).

Ammonium-nitrogen (NH_4-N) concentration was determined in duplicate according to (Weatherburn, 1967). For this, an aliquot of 20 µl of the supernatant obtained after the first step centrifugation for SCFA analysis was pipetted into a 2-mL-vial with the addition of 900 µl of reagent A (2.5 g phenol hypochlorite and 12.5 mg sodium-nitroprusside dissolved in 250 mL distilled water). Subsequently, the mixture was centrifuged at 10,000 × *g* for 10 min, 4°C (Biofuge, Heraeus Holding GmbH, Hanau, Germany). Reagent B ((900 µl; 2.5 g sodium hydroxide + 2.1 mL sodium hypochlorite (containing 12% (v/v) chlorine)) was then added after 4 min, and the mixture incubated at 38°C 20 for min. After incubation, the solution was transferred to a cuvette, and the NH_4-N concentration was read at 625 nm using a spectrophotometer (Varian Cary 50 Bio, UV–vis, Palo Alto, CA, USA).

The method of Boisen & Fernández (1995) modified by Westreicher-Kristen et al. (2013) using the pepsin and pancreatic solubility (PPS) procedure was adopted to determine the *in vitro* intestinal digestibility of DM and CP. For this, the residual pellets obtained after incubation were pooled per experimental diet and incubation period. For each incubation run of the PPS analysis, pooled samples were analyzed in triplicate simultaneously with two blanks containing only incubation medium to correct for the PPS. Pooled samples (400 mg) suspended in a 100 mL conical flask, were thoroughly mixed with 25 mL phosphate buffer (0.1 M; pH 6.0). To the mixture 10 mL of 0.2 M hydrochloric acid was added and its pH was adjusted to 2 using 1 M sodium hydroxide or 1 M hydrochloric acid. Then, 1 mL of pepsin

solution (0.01 g/mL; Merck 7190, 200 FIP U/g) was added to the mixture before it was incubated at 40 °C in an oven for 6 h under constant stirring. Afterwards, 5 mL of 0.6 M sodium hydroxide and 10 mL of phosphate buffer were added, and the pH of the samples was adjusted to 6.8 with 5 M HCl or 5 M sodium hydroxide. Subsequently, 1 mL of freshly prepared pancreatin solution (0.05 g/mL; Sigma P-1750, Sigma-Aldrich, Massachusetts, USA) was added, and the mixture was incubated in an oven at 40°C for 18 h under constant stirring. After incubation, 5 mL of 20% (v/v) of sulfosalicylic acid solution was added to the incubated mixture, which was then left to stand at room temperature for 30 min. Then, the entire contents of the flasks were filtered through a previously weighed filter paper (Whatman paper N° 54, GE Healthcare Life Sciences, Darmstadt, Germany) that was oven-dried at 103°C for 2 h. The insoluble residue in the filter paper was washed with ethanol and acetone, oven-dried again at 103°C for 4 h, and weighed. This insoluble residue was considered to be the apparent *in vitro* intestinal indigestible DM. Finally, the N concentration in this residue was determined by Kjeldahl to calculate the intestinally undigested CP.

The ADIN concentrations of the diets were determined following the standardization procedures for nitrogen fractionation (Licitra et al., 1996).

3.3.5 Protozoa count

For ciliate protozoa count, 1 mL each of the fixated samples was pipetted into two Fuchs-Rosenthal chambers (0.2 mm depth, 2 × 2 mm chamber, 0.25 mm square lined), and ciliate protozoa were counted under 10 × magnification in a microscope (Zeiss, Carl Zeiss Microscopy GmbH, Jena, Germany). The total number of protozoa per mL of fixated sample was calculated from the average counts of the two chambers, and the protozoa count of incubated blank samples was used for correcting the dietary treatment.

3.3.6 Calculations

The non-structural carbohydrates (NSC) concentration was calculated according to the equation of NRC (2001) as follow:

$$NSC = 1000 - (ash + CP + CL + NDF) \quad (1)$$

with NSC, ash, CP, CL and NDF in g/kg DM

The metabolizable energy (ME) of basal ingredients for each diet was estimated using the GfE (2016) equation, and the concentrations of crude nutrients, cumulative gas production (GP)

and ADFom (method 6.5.2 of VDLUFA) for the ME equation were obtained from the silage experiment of chapter 2.

$$ME = 12.49 - (0.0114 \times ADFom) + (0.00425 \times CP) + (0.0269 \times CL) + (0.01683 \times GP) \quad (2)$$

with ME in MJ/kg OM; CP, CL, and ADFom in g/kg OM; and GP in mL/200 mg OM.

$$ME\ (MJ/kg\ DM) = ME\ (MJ/kg\ OM) \times (1000 - CA\ (g/kg\ DM)) / 1000 \quad (3)$$

The ME concentration of corn starch was obtained from Schiemann et al. (1971) and soy protein from Van Eys et al. (2004).

The *in vitro* apparent ruminal DM degradability was calculated as the difference between the substrate DM and the residual dry mass corrected for the residual dry mass from the blanks after *in vitro* incubation and divided by the substrate DM, expressed in percentage. The *in vitro* apparent undegraded CP was calculated from the CP concentrations in the residual substrate after fermentation, corrected for CP concentration in residual substrate recovered from the blanks. It was assumed that all nitrogen determined in the residue originated only from undegraded substrate CP. However, proportions of undegraded CP were likely overestimated due to the attachment of microbial matter to the residual matter/substrate and the contribution of N from the buffer solution. For *in vitro* apparent intestinal DM digestibility calculation, the dried residual pellets corrected for the blank residual dry mass after PPS incubation was subtracted from the residual dry mass after ANKOM incubation and divided by the residual dry mass (ANKOM incubation) expressed in percentage. The *in vitro* apparent total DM digestibility was calculated by multiplying the corrected residual dry mass after ANKOM incubation by the apparent intestinal digestibility coefficient and summed with the difference between the substrate DM and the corrected residual dry mass after ANKOM incubation and then divided by the substrate DM expressed in percentage. *In vitro* apparent intestinal CP digestibility was calculated as the difference between the apparent undegraded CP concentration after ANKOM incubation and the residual CP concentration after PPS incubation divided by the apparent undegraded CP concentration after ANKOM incubation expressed in percentage.

3.3.7 Statistical analysis

Data were analyzed using the GLM procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). The main effect of ensiling length, storage temperature, CP of diets and their interactions for

each incubation period at different sampling hours (n = 6, 2 duplicates × 3 incubations) was analyzed according to the model:

$$Y_{ijk} = \mu + L_i + T_j + P_k + (LT)_{ij} + (LP)_{ik} + (TP)_{jk} + (LTP)_{ijk} + e_{ijk}.$$

where Y_{ijk} = dependent variable, μ = overall mean, L_i = ensiling length effect, T_j = storage temperature effect, P_k = crude protein level effect, $(LT)_{ij}$ = the interaction effect of ensiling length and storage temperature, $(LP)_{ik}$ = the interaction effect of ensiling length and crude protein level, $(LTP)_{ijk}$ = the interaction effect of ensiling length, storage temperature and crude protein level, and e_{ijk} = residual random error of experiment.

For cases in which no interaction effect was observed for any variable, those were not reported. Linear and quadratic effects of ensiling length were determined using orthogonal polynomial contrasts. All significant differences were declared at $P < 0.05$.

3.4 Results

3.4.1 Fermentation parameters

There was no effect of storage temperature on *in vitro* rumen fermentation parameters. In addition, no interaction was found between storage temperature and ensiling length, storage temperature and crude protein level, and the interaction of the three studied factors. Irrespective of the incubation time, GP was quadratically affected by ensiling length (8 h, $P < 0.01$; 24 h, $P = 0.02$; Table 3) with GP increase from 0 to 75 d of ensiling before declining at 180 d. The GP was higher in diets with low rather than high CP concentration at both incubation times ($P < 0.01$; for both incubation times). Besides, the pH was greater in diets with high rather than low CP concentration after 24 h incubation ($P < 0.01$).

The NH_4-N concentrations in the inoculum were greater for all diets after 24 h than after 8 h incubation. An interaction effect between ensiling length and CP level was found for NH_4-N concentrations after 8 h incubation ($P = 0.04$), with greater NH_4-N concentrations for high than low CP diets in all ensiling lengths and with greater absolute difference between CP levels at day 0 than 75 and 180 d. A quadratic response with increasing ensiling length was found for NH_4-N concentration after 24 h ($P < 0.05$) of incubation, increasing the NH_4-N concentration from 40 to 75 d and declining at 180 d. Moreover, after 24 h incubation, the NH_4-N concentration was greater for high than low CP diets ($P < 0.01$).

Counts of ciliate protozoa decreased with advancing ensiling length quadratically ($P = 0.02$) after 8 h incubation with the highest and lowest ciliate protozoa counts at 0 and 75 d, respectively. Besides, there was a linear decrease with increasing ensiling length in counts of ciliate protozoa after 24 h of incubation ($P < 0.01$). The protozoa counts were greater in diets with low than high CP concentration ($P = 0.04$) after 24 h of incubation.

Quadratic responses to prolonging ensiling length were found for total SCFA concentration after 8 h and 24 h of incubation ($P < 0.01$, for both incubation times; Table 4) with an increase in total SCFA concentration from 0 to 75 d of ensiling before declining at 180 d. Similarly, a quadratic effect to increasing ensiling length was found for the acetate proportion, increasing from 0 to 75 d of ensiling before declining at 180 d ($P < 0.01$). Acetate proportion was greater for high than low CP diets after 8 h of incubation. However, after 24 h of incubation, an interaction effect was found between ensiling length and CP level for the acetate proportion ($P < 0.05$) with a greater acetate proportion for low than high CP diets in all ensiling lengths and with a greater absolute difference between CP levels at 180 rather than 75 and 0 d ensiling length. There was also an interaction between ensiling length and CP level for the propionate proportion after 8 h ($P < 0.05$) and 24 h ($P < 0.01$) of incubation. While the propionate proportion was not affected by the CP level at day 0 of ensiling, the propionate proportion was higher in the high CO diets at 75 and 180 d. A linear increase in the isobutyrate proportion with prolonged ensiling length ($P < 0.05$) was found, and the proportion of isobutyrate was greater for low than high CP diets after 8 h of incubation. However, after 24 h of incubation, the isobutyrate proportion increased with advancing ensiling length quadratically ($P < 0.05$) with the highest and lowest propionate proportion at 75 and 0 d ensiling length, respectively. An interaction effect between ensiling length and CP level was found for the butyrate proportion ($P < 0.01$) after 8 h of incubation with a greater butyrate proportion for low than high CP diets and greater total difference between CP levels at 75 and 180 than 0 d ensiling length. The isovalerate proportion increased linearly with ensiling length ($P < 0.01$), and the proportion of isovalerate was lower in high than low CP diets after 8 h of incubation. Moreover, after 8 h of incubation, valerate proportion increased with increasing ensiling length ($P < 0.01$) with a greater proportion of valerate for silages stored indoors rather than outdoor ($P < 0.05$) and a higher proportion for high than low CP diets ($P < 0.01$). Additionally, an interaction effect between ensiling length and CP was found for the proportion of valerate

after 24 h of incubation ($P < 0.01$), with a higher valerate proportion for low than high CP diets and with a greater absolute difference between CP levels at 75 than 180 and 0 d ensiling lengths. The proportion of acetate to propionate ratio after 8 h incubation decreased quadratically with advancing ensiling length ($P < 0.01$) with the highest and lowest proportion at 0 and 75 d ensiling length and a greater proportion of acetate to propionate ratio was found in silages stored outdoors than indoors ($P < 0.05$). Nevertheless, after 24 h of incubation, an interaction effect between ensiling length and CP levels was found for the proportion of acetate to propionate, with a greater proportion for low than high CP diets and a greater absolute difference between CP levels at 75 than 180 and 0 d ensiling length. The proportion of branched chain fatty acids (BCFA) increased linearly with ensiling length after 8 h ($P < 0.01$) and 24 h ($P < 0.05$) of incubation and the CP differed after 8 h of incubation with a greater proportion of BCFA found for low than high CP diets.

3.4.2 In vitro rumen degradability and post-ruminal digestibility

There was no effect of all studied factors on the apparent rumen DM degradability. A quadratic response of apparent intestinal DM digestibility with increasing ensiling length was found after 8 h of incubation ($P < 0.01$ Table 5), whereas it decreases linearly after 24 h of incubation ($P < 0.01$). Similarly, the apparent total DM digestibility after 24 h incubation decreased linearly ($P < 0.01$) with increasing the ensiling length,

The amount of CP concentration in the undegraded substrate after 8 h and 24 h of incubation ($P < 0.01$; for both incubation times) decrease linearly with advancing ensiling length. Additionally, the amount of CP concentration in the undegraded substrate was greater in high than low CP diets after 8 h and 24 h of incubation ($P < 0.01$ for both incubation times).

There was an interaction effect between ensiling length and storage temperature for apparent intestinal CP digestibility after 8 h ($P = 0.02$) and 24 h ($P < 0.01$) of incubation. The apparent intestinal CP digestibility was greater for silages stored outdoors than indoors at 75 d and lower for silages stored outdoors than indoors at 180 d after 8 h incubation and with no difference between storage temperature at 75 d and 180 d. Besides, the apparent intestinal CP digestibility was greater for indoor than outdoor storage at 75 d and 180 d after 24 h of incubation and with a greater absolute difference between storage temperatures at 180 d than 75 d. Moreover, after 8 h and 24 h of incubation, apparent intestinal CP digestibility was greater ($P < 0.01$ for both incubation times) in diets with high than with low CP concentration.

3.5 Discussion

3.5.1 In vitro rumen fermentation

The anaerobic microbial fermentation end products in the rumen are gases, methane, ammonia and most importantly, SCFA, which provides ruminants with a major source of metabolizable energy, and is considerably influenced by diet (Bergman 1990). Increasing the CP level of diets reduced the GP, and the GP response to the increasing ensiling length was quadratic, with the highest GP at around 75 d of ensiling length. Previous studies (Cone et al. 2009; Cone and van Gelder 1999; Steingass 1983) have shown that the contribution of protein fermentation to GP is negligible compared to carbohydrate fermentation which is consistent with the finding in the present study. The greater concentration of NSC in low CP diets and in diets from forages ensiled at 75 d that were rapidly fermented likely enhanced fibre degradation thereby increasing the GP. Although no differences were found across diets for DM degradability in the present study, it appears that energy supply and availability of ruminal N increased microbial growth and activities which positively influences GP (Castro et al. 2021; Cone and van Gelder 1999; Zhang et al. 2016). Besides, the greater GP from diets with silages stored for 75 d compared to those ensiled for 180 d and as compared to the diets from fresh forages may be associated with the greater availability of ruminal N that promoted rumen microbes production thereby increasing fibre degradation. Moreover, the fibre concentrations of silages stored for 75 d may be more degradable, indicating lower usage of readily fermentable carbohydrates by silage microbes during ensiling at 75 d compared to 180 d.

Table 3. Effect of ensiling length (L), storage temperature (T) and crude protein (CP) level on gas production (GP), pH, ammonia-N (NH_3-N) and total ciliate protozoa count in rumen inoculum at different incubation periods (least square means; n = 6)

Ensiling length		0 d		75 d				180 d				SEM	*P* – value				
Storage temperature				25°C		30°C		25°C		30°C			Ensiling length				
CP levels (g/kg DM)		90	130	90	130	90	130	90	130	90	130		Lin	Quad	T	CP	L*CP
Variables	Time																
GP (mL/g DM)	8 h	77.5	67.0	88.8	64.1	91.6	80.6	71.3	57.4	68.6	58.2	4.06	<0.01	<0.01		<0.01	
	24 h	151	149	176	132	174	160	157	132	150	141	8.38		0.02		<0.01	
pH	8 h	6.74	6.73	6.73	6.72	6.73	6.73	6.74	6.74	6.74	6.74	0.01					
	24 h	6.62	6.67	6.63	6.68	6.40	6.66	6.66	6.66	6.66	6.68	0.02				<0.01	
NH_4-N (mg/L)	8 h	20.6	25.5	26.4	27.5	27.4	28.3	26.0	25.7	23.7	25.3	1.28		<0.01		<0.01	0.04
	24 h	33.0	39.3	40.0	44.2	38.9	43.0	37.1	42.4	37.8	41.2	2.52		0.01		<0.01	
Protozoa ($\times 10^3$/mL)	8 h	6.21	5.38	3.85	2.27	3.23	3.48	3.17	2.10	4.74	4.00	1.06	<0.01	0.02			
	24 h	7.71	5.93	5.84	3.86	5.60	4.96	5.14	4.33	4.55	4.02	1.02	<0.01			0.04	

SEM, standard error of means; Lin, Linear; Quad, Quadratic; L*CP, interaction effects of ensiling length with crude protein level

L*T, interaction effects of ensiling length with storage temperature ($P > 0.1$); CP*T, interaction effects of crude protein level with storage temperature ($P > 0.1$)

L*CP*T, interaction effects between ensiling length, crude protein level and storage temperature ($P > 0.1$)

Table 4. Effect of ensiling length (L), storage temperature (T) and crude protein (CP) level on the in vitro fermentation short-chain fatty acid (SCFA) concentration and individual SCFA proportion at different incubation periods (least square means; n = 6)

Ensiling length		0		75		75		180		180		SEM	*P* – value				
Storage temperature				25°C		30°C		25°C		30°C			Ensiling Length				
CP levels (g/kg DM)		90	130	90	130	90	130	90	130	90	130		Lin	Quad	T	CP	L*CP
Variables	Time																
Total SCFA[1]	8 h	29.6	29.6	32.6	31.9	32.0	31.6	31.6	30.4	29.9	29.1	0.57		<0.01	0.06		
(μmol/mL)	24 h	44.6	44.0	47.0	45.8	47.0	46.2	45.7	44.1	44.6	44.0	0.93		<0.01		0.08	
Individual SCFA proportions (μmol/mol total SCFA)																	
Acetate	8 h	68.2	69.1	64.4	64.7	65.0	65.6	65.1	65.7	65.5	66.3	0.42	<0.01	<0.01		<0.01	
	24 h	65.5	65.8	63.7	62.0	64.0	62.3	64.3	62.4	64.5	62.6	0.56	<0.01	<0.01		<0.01	<0.05
Propionate	8 h	17.5	17.2	19.2	21.3	19.0	20.8	18.7	20.4	18.5	20.2	0.68	<0.01	<0.01		<0.01	<0.05
	24 h	18.4	18.4	19.6	21.6	19.0	21.4	19.3	21.2	19.3	21.1	0.42	<0.01	<0.01		<0.01	<0.01
Iso-butyrate	8 h	0.91	0.90	1.02	0.91	1.00	0.89	1.03	0.92	1.03	0.92	0.04	<0.05			<0.01	0.08
	24 h	0.99	1.01	1.07	1.03	1.10	1.04	1.07	1.06	1.04	1.04	0.02	<0.05	<0.05			
Butyrate	8 h	11.6	11.0	13.1	10.9	13.0	10.7	12.9	10.9	12.8	10.6	0.39				<0.01	<0.01
	24 h	12.6	12.3	12.6	12.6	13.0	12.5	12.3	12.6	12.3	12.5	0.33					
Iso-valerate	8 h	0.93	0.92	1.21	1.01	1.20	0.96	1.24	1.02	1.20	0.97	0.08	<0.01	<0.05		<0.01	0.07
	24 h	1.45	1.48	1.68	1.55	1.70	1.54	1.67	1.55	1.59	1.53	0.08					
Valerate	8 h	0.85	0.88	1.03	1.14	1.00	1.09	0.99	1.08	0.97	1.04	0.02	<0.01	<0.01	<0.05	<0.01	
	24 h	1.06	1.12	1.32	1.23	1.30	1.22	1.27	1.19	1.23	1.17	0.03	<0.01	<0.01		<0.05	<0.01
C2:C3	8 h	3.89	4.05	3.39	3.04	3.48	3.17	3.52	3.23	3.58	3.29	0.29	<0.01	<0.01	<0.05		
	24 h	3.60	3.60	3.25	2.87	3.35	2.91	3.34	2.94	3.35	2.97	0.34	<0.01	<0.01		<0.01	<0.05
(C2+C4):C3	8 h	4.62	4.70	4.07	3.55	4.18	3.67	4.22	3.76	4.29	3.82	0.38	<0.01	<0.01	0.08	0.07	0.09
	24 h	4.29	4.26	3.89	3.46	3.99	3.50	3.97	3.54	3.99	3.56	0.37	<0.01	<0.01		<0.01	<0.05
Total BCFA	8 h	1.83	1.82	2.23	1.92	2.30	1.84	2.27	1.94	2.24	1.89	0.12	<0.01			<0.01	
	24 h	2.45	2.49	2.76	2.58	2.70	2.59	2.74	2.61	2.63	2.57	0.10	<0.05				

[1]Total SCFA corrected for the SCFA concentrations in the inoculum of the blank bottle at each incubation sampling time, C2:C3, Acetate: Propionate; (C2+C4):C3, Acetate + Butyrate: Propionate, BCFA, branched chain fatty acids (isobutyrate + isovalerate), Lin, Linear; Quad, Quadratic; SEM, standard error of means, L*T, interaction effects of ensiling length with storage temperature ($P > 0.1$), CP*T, interaction effects of crude protein level with storage temperature ($P > 0.1$), L*CP*T, interaction effects between ensiling length, crude protein level and storage temperature ($P > 0.1$)

Table 5. Effect of ensiling length, storage temperature and crude protein level on the in vitro degradability and post-ruminal digestibility of diets dry matter and crude protein at different incubation periods (least square means; n = 6)

Ensiling length		0 d		75 d				180 d				SEM	*P*-value				
Storage temperature				25°C		30°C		25°C		30°C			Ensiling length				
CP levels (g/kg DM)		90	130	90	130	90	130	90	130	90	130		Lin	Quad	T	CP	L*T
Variables	Time																
Apparent ruminal DM	8 h	40.7	42.8	45.8	46.6	42.9	45.6	47.2	45.6	45.3	44.7	4.52					
degradability (g/100 g)	24 h	49.7	48.7	50.2	48.2	50.3	48.9	50.6	45.7	46.7	45.7	1.76	0.09				
Apparent intestinal DM	8 h	48.3	47.9	48.3	48.6	48.8	50.0	46.1	46.5	45.4	44.0	0.85	<0.01	<0.01			
digestibility (g/100 g)	24 h	45.0	46.8	45.7	45.9	43.9	44.8	41.8	38.8	38.7	39.1	1.04	<0.01	<0.01			
Apparent total DM	8 h	66.0	67.1	67.4	68.2	67.2	68.8	67.0	66.9	64.7	65.0	2.91					
digestibility (g/100 g)	24 h	72.3	72.7	72.9	71.9	72.1	71.7	71.2	66.8	67.3	66.8	0.97	<0.01	0.01			
CP in undegraded	8 h	205	235	184	213	192	211	150	187	146	186	12.5	<0.01			<0.01	
substrate (mg CP)	24 h	179	208	166	206	172	202	142	186	148	178	6.14	<0.01			<0.01	
Apparent intestinal CP	8 h	71.2	74.9	73.0	74.7	75.9	76.9	76.2	77.4	73.5	75.1	1.26	<0.01			<0.01	0.02
digestibility (g/100 g)	24 h	74.2	75.5	70.9	72.4	70.8	72.0	71.3	71.9	66.8	66.5	0.80	<0.01	0.02	<0.01	<0.01	<0.01

DM, dry matter; SEM, standard error of means

Lin, Linear; Quad, Quadratic; L, ensiling length; T, storage temperature; CP, crude protein level;

L*CP, interaction effects of ensiling length with crude protein level ($P > 0.1$), CP*T, interaction effects of crude protein level with storage temperature ($P > 0.1$)

L*CP*T, interaction effects between ensiling length, crude protein level and storage temperature ($P > 0.1$)

Accordingly, the total SCFA concentrations in rumen inoculum increased quadratically with increasing ensiling length and with a tendency for the effect of CP level. The tendency for higher total SCFA concentrations with a low CP diet is consistent with the high GP and the decrease in the rumen inoculum pH. This might be attributed to the greater NSC concentration and greater digestion supplying a more fermentable substrate for rumen fermentation. Additionally, the action of silage inoculant on preserving NSC of forages ensiled at 75 d rather than 180 d could have enhanced fibre accessibility by rumen microbes better than other ensiling lengths (González et al. 2007), thereby resulting in greater total SCFA concentrations as some studies have reported improvement in DM and fibre digestibility of silage treated with mixed bacterial inoculant (Addah et al. 2011, 2012) such as was used during ensiling in the previous silage study (Aloba et al. 2022). The higher fibre and ADIN concentration in forages ensiled at 180 d than 75 d as associated with the reduction of soluble carbohydrates during ensiling (Aloba et al. 2022) could be attributed to the decline of total SCFA concentration in the rumen with diets from forages ensiled for 180 d.

Moreover, the interaction of ensiling length and CP levels stimulated varying shifts in the profile of individual SCFA proportion in rumen inoculum. The interaction between ensiling lengths and CP levels is a reflection of the higher ratio of NSC to ADF concentration in the diets from forages ensiled at 75 d, as it influences the shift in the ratio of acetate to propionate. Additionally, the supplemented CP in high CP diets provided rumen fermentation with additional hydrogen sinks thereby increasing the propionate level.

The relatively high proportion of propionate in the rumen inoculum with increasing ensiling lengths agrees with other studies showing that lactate in silages is predominantly fermented into propionate in the rumen (Jaakkola and Huhtanen 1992, 1993; Lima et al. 2010). Besides, the enhanced propionate proportion in high CP diets is likely related to the higher proportion of grain ingredients in high CP diets, which have typically been reported to increase propionate proportion (Agle et al. 2010).

Butyrate is primarily produced by protozoa (Morgavi et al. 2012), as consistent with higher protozoal counts in inoculum from diets with lower CP concentrations in the present study. Equally, there is a positive correlation between protozoal populations and increased starch concentration (Dijkstra 1994) and this was observable in the current study. Furthermore, starch is an essential substrate to protozoans (Morgavi et al. 2012). On the contrary, holotrich

protozoa have limited ability to degrade structural carbohydrates (Williams and Coleman 1997). These protozoa species may have constituted most protozoa counted in the present study, which reduces with higher fibre concentration related to increasing ensiling length.

3.5.2 Ruminal degradation and post-ruminal digestibility

There was no difference across diets for ruminal DM degradation but an increase in DM degradation with increasing incubation time was observed. The increase in the availability of N for microbial growth and the time provided for rumen microbes to attach and degrade diets may be responsible for the increased DM degradation at 24 h of incubation. However, there was a linear decrease in the digestibility of undegraded DM post-ruminally with increasing ensiling length. This indicates that with increasing ensiling length, the fibre component of the diets was less digested by rumen microbes.

Primarily, ruminal CP degradation is affected by protein solubility, interaction with other nutrients, and the predominant microbial population (Bach et al. 2005). The NH_4-N concentrations in rumen inoculum were greater with high rather than low CP concentration for all diets and at both incubation times. This observation is due to the greater CP concentration for high than low CP diets as soy-protein was included in the high CP diet composition suggesting that dietary CP level plays a major role in ruminal protein degradation. Similarly, Dung et al. (2014) found a greater NH_4-N concentration in rumen inoculum with increasing dietary CP from 100 g to 190 g/kg DM. Moreover, the NH_4-N concentrations in rumen inoculum increased quadratically with advancing ensiling length showing greater CP degradation. The variation between the proportion of true protein in silages at 75 d and 180 d that originated from the increase in protein degradation to NH_3 during ensiling with increasing ensiling length in our previous study (Aloba et al. 2022) may be related to this quadratic response.

The concentration of CP in the undegraded substrate decreased linearly with increasing length suggesting higher substrate CP degradation from silage diets than fresh forage diets. This observation may be explained by the increased soluble CP in silages, consistent with findings from previous studies on silages (Broderick 2018; Givens and Rulquin 2004; Jaakkola and Huhtanen 1993). Besides, the rate of degradation of non-protein nitrogen and soluble CP from silages in the rumen is high (Givens and Rulquin 2004), and this was reflected in the higher proportion of valerate and concentration of ruminal NH_4-N produced from silage diets

rather than from fresh forage diets in the present study. Overall, the likely overestimation of the amount of CP in the undegraded substrate in the current study might be due to the rumen fluid's microbial mass nitrogen contribution.

The fibre-bound protein proportion in both soybean and sorghum silages increased with ensiling length and was greater for outdoors storage temperatures than indoor during ensiling in our previous study (Aloba et al. 2022). Thus, the decline in apparent intestinal CP digestibility with increasing ensiling length and at outdoor storage may be related to the considerable reduction in the soluble CP fraction, leading to the increase in the proportion of the indigestible CP fraction in the total CP of particulate matter (Cone et al. 2006). It is well established that the slowly degraded CP fraction (B_3) of feed escapes ruminal degradation thereby making it available for digestion in the lower gut (Licitra et al. 1996). Accordingly, the decline in the apparent intestinal CP digestibility with increasing ensiling length may be related to the decrease in the proportion of B_3 that was mediated by the rise in the ADIN proportion of the silages stored outdoors with increasing ensiling length from our previous study, considering that the proportion of forage in the diet contributed more to the indigestible CP fraction.

Lima and colleagues (Lima et al. 2011) observed a positive effect of ensiling on sorghum-soybean forage mixtures. In that study, forage ensiled between 162-182 d showed a higher proportion of intestinal digestible CP although the authors did not provide details of the causal factors. Additionally, the apparent intestinal CP digestibility increased with diet CP concentration in the present study. Previous studies have reported an apparent intestinal CP digestibility of 98% for soy protein using the modified three-step procedure (Boucher et al. 2009), higher than the 93% assumption of the NRC (NRC 2001) model. Therefore, the proportion of soy protein in the diet with high CP concentration might have contributed more to the increase in the intestinal CP digestibility than the forage or silage proportion apart from the likely overestimation of the intestinal CP digestibility due to the rumen fluid's microbial mass nitrogen contributions. Although correction for microbial CP for substrate residues after the *in vitro* incubation to quantify the CP contribution of microbial origin was not done, the contribution of the silage CP proportion in the residue might be low, given that the acid detergent insoluble nitrogen (ADIN) and ADF concentration of sorghum and soybean silage are higher than that of soy protein.

3.6 Conclusions

The results of this study demonstrates that ensiling length had a greater impact on silage rumen fermentation and post-ruminal CP digestibility than storage temperature. Even though the effect of the interaction of silages and CP level on rumen fermentation and post-ruminal CP digestibility followed the same pattern as the interaction of fresh forages and CP level, it became evident that ensiling of forages until 75 d increased the end products of microbial fermentation in the rumen compared to fresh forages and prolonged storage beyond 75 d. Increasing the length of ensiling and CP of diets enhances CP ruminal degradation. However, ensiling beyond 75 d reduces CP digestibility to an extent that cannot be recovered by supplying additional CP. Finally, higher temperature of silage stored outdoor negatively influenced the CP intestinal digestibility.

3.7 References

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Chapter 4

4 Nutrient intake, digestibility, protein metabolism, and performance of growing steers fed corn or corn-soybean silage at different crude protein levels

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Temitope Alex Aloba, Elmer Edgardo Corea, Manuel Mendoza, Mizeck Chagunda, Uta Dickhoefer and Joaquin Castro-Montoya. 2022. Nutrient intake, digestibility, protein metabolism and performance of growing steers fed corn or corn-soybean silage at different crude protein levels.

4.1 Abstract

The study aimed to investigate the effects of crude protein (CP) levels, silage type, and their interaction on nutrient intake, nutrient digestibility, methane concentration, protein metabolism, and performance in growing steers. Sixteen growing tropical crossbred steers were assigned to a 4 × 3 incomplete Latin square experimental design comprising 17 d periods, each with 12 d of adaptation to dietary treatments and 5 d of sampling. The dietary treatments were arranged in a 2 × 2 factorial design with two main silages (corn silage (CS) and corn-soybean silage (CSS); 60:40 dry matter [DM] basis of corn to soybean) offered at two dietary CP levels each [115 g/kg of DM (low CP) and 135 g/kg of DM (high CP)]. At each sampling period, the live weight of steers was measured, and samples of feed, faeces, urine, and blood were obtained and analysed. The main effects of CP, silage type, and their interactions were tested using PROC GLIMMIX in SAS 9.4. There was no effect of both CP concentration and silage type on nutrient intake. Still, the apparent total tract digestibility of nutrients was lower for low than high CP concentrations in both silages, with greater differences between the CP levels in CS than CSS ($P < 0.01$). Similarly, high CP concentration increased nitrogen (N) intake, urinary N excretion, and blood urea-N concentration in both silages when compared to low dietary CP concentration ($P < 0.01$) but reduced N retention in CSS while increasing it in CS ($P < 0.01$). Methane concentration in growing steers was lower at high than at low dietary CP concentration with CSS but greater in diets with high CP concentration containing CS ($P < 0.05$). The average daily gain and feed conversion efficiency of steers were greater in CS diets with low than high CP concentration ($P < 0.05$) but similar in CSS diets irrespective of their CP concentration. Increasing the CP concentration, regardless of the silage type, did not improve performance, suggesting the low CP concentration was optimal for growing tropical crossbred steers.

Keywords: forage legume; methane; silage; steers; protein metabolism

4.2 Introduction

The proportion of forage conserved as silage in the tropics has recently increased (Wilkins and Wilkinson, 2015), with whole-crop cereal silages (mainly corn) becoming the primary forage source for cattle diets in intensive operations due to their high yield, energy richness, and good silage quality (Khan et al., 2015). However, the crude protein (CP) concentrations of

whole-crop cereal silages are generally low (Huuskonen et al., 2014) despite sufficient fermentable substrates, thus necessitating protein supplementation. Under these circumstances, the utilisation of forage legume silage as a protein source in cattle feeding may be a valuable option for increasing the efficiency of nutrient utilisation and reducing the share of imported feeds, which are partially associated with high carbon footprints (Bureenok et al., 2016; Jaramillo et al., 2021; Nkosi et al., 2016). Yet, the ruminal degradation rate of CP from legume silages is typically high, which may reduce the efficiency of microbial crude protein (MCP) synthesis (Givens and Rulquin, 2004). Hence, complementing whole-crop cereal silages with forage legume silages might stimulate ruminal MCP synthesis and thereby enhance CP supply, feed intake, nutrient digestibility, and performance of growing cattle during the dry season.

In most silage feeding studies evaluating mixtures of cereal crops or grasses with forage legumes, these forages were ensiled individually, and the proportion of legume silage to cereal crop or grass silage in the mixed ration is either increased or decreased during feeding evaluation (Hassanat et al., 2014; Schulz et al., 2018; Souza et al., 2014). However, the nutritional quality of silage mixtures from cereal crops and forage legumes when ensiled together differ compared to when ensiled individually due to silage fermentation processes, as found in a previous silage study (Aloba et al., 2022a). Remarkably, the ensiling conditions over time and the physicochemical properties of forage at ensiling influence the proportion of protein fraction, which affects protein availability ruminally and post-ruminally (Aloba et al., 2022b). In this context, supplementing such silage with another CP source may enhance performance and feed efficiency. However, the quantity and quality of CP and its contribution to meeting rumen microbes and cattle demands are variable. Thus, CP requirements are influenced by protein metabolism, making it complex to estimate the actual CP requirements of beef cattle. In this line, studies investigating the effects of dietary CP concentrations have shown contrasting results in growing beef cattle performance with increasing CP concentrations (Amaral et al., 2018; Amorim et al., 2020; Menezes et al., 2016).

While the inclusion of forage legume silage complements whole-crop cereal silages, changes in silage type may likely associate differently with different levels of CP, affecting nutrient supply. Therefore, the study aimed to understand the interaction effects of dietary CP concentration and type of silage (i.e., whole crop silage from corn alone or corn and soybean)

on feed intake, nutrient digestibility, protein metabolism, and growth performance of growing steers. It was hypothesised that feed intake, nutrient digestibility, rumen MCP synthesis, and steer performance would increase at high dietary CP concentration but that the effects of low dietary CP concentration would be maximised in corn-soybean silage (CSS) than corn silage (CS) diet.

4.3 Materials and methods

The study comprised forage establishment, silage preparation, and an *in vivo* feeding experiment conducted at the experimental research station of the University of El Salvador, San Luis Talpa, El Salvador (13°28′19″N, 89°05′59″W, at 62 m above sea level). Forages were cultivated and silages prepared between August 2019 and March 2020, whereas the *in vivo* feeding experiment was conducted between March and April 2020. The animal experiment was approved by the University of El Salvador animal research ethics and welfare committee.

4.3.1 Forage Cultivation and Ensiling

Corn (*Zea mays;* CENTA H-59) and soybean (*Glycine max;* SIATSA-194) were sown in pure stands with the aid of a planter (John Deere, Moline, Illinois, USA) on 0.8 ha on 22nd August 2019 and 0.5 ha area on 3rd September 2019, respectively. Before sowing, corn and soybean seeds were treated with Blindage®, a seed protectant (Bayer AG GmbH, Leverkusen, Germany). At sowing, 100 kg/ha of ammonium sulphate fertiliser was applied for both forages. Corn was harvested 83 d and soybean 70 d post sowing using a forage harvester (Pecus CX, NB Maaquinas, Itapira, Brazil) equipped with a chopper. Both forages were harvested at a cutting height of 5 cm and chopped to a length of approximately 20 mm. Corn was combined with soybean at a ratio of 60 to 40 (on a dry matter (DM) basis) after harvest and ensiled in piles to create a mixed corn-soybean whole-crop silage. Besides, whole-crop corn was also ensiled separately in piles. Silages were prepared without the use of any additives and were stored for 132 d until the start of the feeding trial.

4.3.2 Experimental Design, Animals, and Feeding

The experiment was designed as a 4 × 3 incomplete Latin square design with a 2 × 2 factorial arrangement of dietary treatments. Each period comprised 12 d of adaptation to the experimental diets and 5 d of sampling and data collection/measurements.

A total of 16 clinically healthy, crossbred (brown swiss × creole) beef-growing steers were selected and housed individually in metabolic cages positioned in a free-stall barn for the experiment. Upon selection, the steers were weighed on two consecutive days at the start of the experiment, and the average live weight (LW) was used as the initial LW (arithmetic mean 155 kg, one SD 25.4 kg). Steers were divided into four treatment groups based on this initial LW and were randomly allocated to each group. They were weighed once daily on two consecutive days at the beginning of each period and at the end of the entire experiment prior to morning feeding to determine their average daily LW gain (ADG).

Four dietary treatments were created in a 2 × 2 factorial design (i.e., two types of silages and two levels of dietary CP concentrations). The four groups of steers were randomly assigned to one of the four dietary treatments, such that each steer received three diets throughout the entire experiment. The dietary treatments were fed as total mixed rations (TMR) composed of 60% of either CS or CSS, 10% star grass hay, and 30% concentrate on a dry matter basis with dietary CP concentrations of 115 g (i.e., low CP) or 135 g /kg DM (i.e., high CP). The concentrate mixture in the TMR was composed of soybean, corn, wheat bran, sugarcane molasses, bypass fat, mineral salt, and mineral-vitamin mixture. All diets were formulated using CPM dairy V3.08 to meet the nutrient requirements for CP and energy, of growing beef steers weighing 160 kg LW, according to NRC (2001). The chemical composition of the forages and the ingredient composition of the four diets are shown in Tables 1 and 2.

The TMR was offered twice daily (9.00 and 16.00 h) throughout the study, and all steers had ad libitum access to fresh drinking water. The amount of TMR allocated to each steer each day was calculated to equal 3% of its LW on a DM basis at the beginning of each period. Samples of silage and hay were collected weekly to determine DM for the adjustment of feed offered. The daily feed intake of individual steers was recorded throughout the sampling period, and refused diets were collected and weighed daily before the morning feeding on subsequent days. Diets offered, diets refused, and individual feed ingredients were sampled daily and stored at -20°C. After each period, samples were pooled by weight, and the pooled samples were stored at -20°C until analysis.

4.3.3 Urine and faeces collection

For quantitative total daily urine collection, steers were fitted with harnesses and urine collectors that were designed by the Department of Animal Nutrition and Rangeland Management in the Tropics and Subtropics of the University of Hohenheim, Germany. The fitted harnesses were attached to urine collectors with a hose, where urine was channelled into plastic containers. Each container was prefilled with 20 mL of 98% (v/v) sulfuric acid to lower the urine pH below 3, preventing microbial degradation of purine derivatives (PD) and ammonia losses (Chen and Gomes, 1992). Once daily, the total urine excreted per steer was homogenised, urine volume was recorded, and a sample of 100 mL was filtered through a surgical gauze to remove solid materials. A subsample of 50 mL was stored at -20°C until the end of the experiment, whereas the remaining 50-mL-subsample was filtered through a filter paper with 250 mm pore size and diluted with distilled water at the ratio of 1:5 (v/v). The diluted urine was thoroughly mixed and stored at -20°C until the end of the experiment.

At the end of the experiment, daily diluted and undiluted urine samples were thawed and pooled per steer and period. The amount of urine obtained from each daily urine excretion sample for the pooled samples correspond to the proportion of urine volume of the total urine excretion for each steer during the sampling period. Pooled samples of diluted and undiluted urine were stored at -20°C for PD and nitrogen (N) analysis, respectively.

Total faecal output was collected daily by scraping faeces from the floor every 2 h from 8.00 to 22.00h and every 3 h thereafter. Faeces were weighed and thoroughly mixed, and about 250 g subsample was stored at -20°C until pooling. At the end of the experiment, daily faecal samples were thawed and pooled by steer and period. The amount of faeces obtained from each daily faecal excretion sample for the pooled samples corresponds to the proportion of faeces of the total faecal excretion for each steer during the sampling period. Composite faecal samples were stored at -20°C for later analysis.

4.3.4 Blood sampling

Blood of all steers was sampled from the jugular vein before and after morning feeding on the 2nd and 4th day of each sampling period, using a jugular catheter. About 10 mL of blood samples were collected into heparinised tubes and centrifuged at 3000 × g for 10 min. Following centrifugation, plasma was collected into scintillation vials and stored at −20°C until analysis.

Table 1. Chemical composition of forages (g/kg dry matter) fed to growing steers (expressed as means ± standard deviation between periods, n = 3)

Variables	Corn silage	Corn-soybean silage	Star grass hay
Dry matter (g/kg fresh matter)	259 ± 2.22	269 ± 2.70	858 ± 1.09
Crude ash	73.2 ± 0.95	94.2 ± 0.44	89.9 ± 0.94
Crude protein	108 ± 2.15	167 ± 1.77	43.5 ± 0.69
Neutral detergent fibre	469 ± 2.15	475 ± 2.32	634 ± 1.43
Acid detergent fibre	271 ± 1.58	313 ± 0.80	370 ± 1.12
Acid detergent lignin	41.3 ± 0.13	68.2 ± 1.43	43.5 ± 0.69

Table 2. Ingredient and chemical composition of dietary treatments (expressed as means ± standard deviation between periods, n = 3) fed to growing steers (n =18)

Items	Silage type			
	Corn silage		Corn-soybean silage	
	CP-115	CP-135	CP-115	CP-135
Ingredients (g/kg)				
Corn silage	592	591	-	-
Corn-soybean silage	-	-	592	592
Star grass hay	99.9	99.6	99.8	99.8
Soybean meal	86.7	134	-	37.3
Ground corn grain	105	64.0	231	165
Wheat bran	78.7	68.4	36.6	72.7
Sugarcane molasses	16.9	16.9	26.1	16.9
Mineral-vitamin mixture[1]	8.90	8.80	8.90	8.90
Mineral salt	5.90	5.90	5.90	5.90
Bypass fat[2]	5.90	11.8	-	2.00
Chemical composition of diets				
Dry matter (g/kg)	324 ± 1.69	331 ± 0.10	345 ± 0.05	346 ± 1.74
Organic matter	904 ± 1.04	909 ± 1.13	907 ± 0.31	908 ± 0.52
Crude protein	118 ± 0.44	135 ± 0.02	121 ± 0.09	138 ± 0.03
Starch	56.6 ± 0.60	46.8 ± 1.16	71.2 ± 1.16	59.6 ± 1.39
Neutral detergent fibre	468 ± 0.54	463 ± 0.61	452 ± 0.25	458 ± 2.01
Acid detergent fibre	254 ± 0.27	253 ± 0.78	263 ± 0.56	266 ± 1.71
Acid detergent lignin	35.9 ± 0.44	33.6 ± 0.37	47.3 ± 0.68	50.5 ± 0.20
Crude lipid	25.0 ± 0.08	28.4 ± 0.06	32.2 ± 0.38	37.9 ± 0.16
Metabolizable energy (MJ/kg)	8.94 ± 0.98	8.62 ± 0.14	8.73 ± 0.04	8.89 ± 0.01
NE_m (MJ/kg)[3]	7.70 ± 0.08	7.16 ± 0.05	7.31 ± 0.18	7.53 ± 0.11
NE_g (MJ/kg)[4]	5.96 ± 0.07	5.48 ± 0.10	5.60 ± 0.16	5.79 ± 0.15

[1] NutroKel® (per kg DM): 8.5 g calcium, 5.6 g phosphorus, 1.6 g magnesium, 0.4 g sulfur, 80 mg copper, 40 mg Iron, 241 mg Zinc, 2.8 mg Selenium, 5100 IU Vitamin A, 4020 IU Vitamin D, and 141 IU VitaminE

[2] Lactomil®: palm oil = 850 g/kg, calcium 80-96 g/kg, and 24.18 MJ Nel /kg (as-fed basis).

[3] Net energy maintenance (MJ/kg) = $1.37 \times ME - 0.138 \times ME^2 + 0.0105 \times ME^3 - 1.12$ (NRC, 2000)

[4] Net energy gain (MJ/kg) = $1.42 \times ME - 0.174 \times ME^3 - 1.65$ (NRC, 2000).

4.3.5 *Methane measurement*

The concentration of methane in ppm produced by each steer was measured using a hand-held laser methane detector (LMD; Tokyo Gas Engineering Solutions Corporation, Tokyo, Japan). Methane measurements were taken at 5.00, 11.00, 14.00, and 17.00 h for 4 min on the 1st, 3rd, and 5th day of each sampling period. The protocol and principle behind the usage of LMD in livestock experiments have been extensively described (Chagunda, 2013; Ricci et al., 2014). For the measurement, a distance of 1 m was maintained between the LMD and the steers' nostrils, and methane concentration was recorded every 0.5 s within a 4-min-time frame per steer and point of time. The 1-m-distance was used to convert the unit of the methane concentration from ppm-m to ppm (Ricci et al., 2014).

4.3.6 *Laboratory analysis*

Pooled samples of offered and refused feeds, individual diet ingredients, and faeces were analysed in duplicates according to the analytical methods of VDLUFA (2012). The samples were thawed and oven-dried at 65°C for 48 h. Subsequently, samples were ground through a 1-mm-screen in a Wiley mill (Thomas Scientific, Swedesboro, USA) and stored for further analysis. Dried samples were analysed for DM (method 3.1) and crude ash (method 8.1) to determine OM concentrations. The neutral detergent fibre (NDF, method 6.5.1), acid detergent fibre (ADF, method 6.5.2), and acid detergent lignin (ADL, method 6.5.3; all with residual ash) were analysed sequentially using Ankom 200 Fiber Analyzer (Ankom Technology, Fairport, New York, USA). Heat-stable amylase and sodium sulphite were used for NDF analysis. Total N concentration of the offered and refused feeds, individual diet ingredients, faeces, and acidified urine samples were analysed using the macro - Kjeldahl method (method 4.1.1), and crude protein (CP = N × 6.25) concentrations were calculated. Pooled samples of the offered feeds were also analysed for crude lipid using the ether extraction procedure (method 5.1.1), and starch analysis was conducted by polarimetry (method 7.2.1). Additionally, triplicate samples of offered and refused feeds were incubated *in vitro* for 24 h in three independent runs on separate days following Menke et al. (1979) procedures to estimate their metabolisable energy (ME) concentrations from *in vitro* gas production.

The pooled samples of undiluted urine were analysed for PD (i.e., allantoin and uric acid) following the modified procedure of Balcells et al. (1991) and Chen and Gomes (1992) and for

creatinine according to the modified procedure of George et al. (2006) using an ultraviolet spectrophotometer (Shimadzu, UV-1280, Kyoto, Japan).

Using an enzymatic colourimetric method (COBAS C 501 Module, Roche Diagnostics, Mannheim, Germany), blood samples were analysed for blood-urea-nitrogen (BUN), total protein, calcium, and phosphorus at Alteclab (Santa Tecla, El Salvador).

4.3.7 Calculation

For the daily estimation of each steer DM intake (DMI), the DMI of the ingested feed was quantified as the difference between the amount of feed offered and refused (on a DM basis), and the mean value across the 5 d sampling period was determined. Similarly, OM, CP, NDF, and ADF concentrations of ingested diets were calculated. For digestible OM intake (DOMI) calculation, daily faecal OM excretion of steers was subtracted from OM intake. The feed efficiency was calculated as the ratio of DMI to ADG of respective steers.

The apparent total tract digestibility (ATTD) of DM, OM, CP, NDF, and ADF was calculated from the difference between the steers' DM, OM, CP, NDF, and ADF intakes and their faecal DM, OM, CP, NDF, and ADF excretions.

Urinary PD (i.e., the sum of allantoin and uric acid) and creatinine excretions (mmol/steer) were calculated by multiplying the urine volume (L/ steer/ period) with the PD or creatinine concentration (mmol/L) in the urine of each steer per period. The ratios of urinary concentrations of PD to creatinine, PD and N, and PD to creatinine index were calculated as indicators for rumen N turnover (Chen et al., 1995). The model proposed by Verbic et al. (1990) was used to calculate the duodenal absorption of microbial PD (mmol/ steer and period), while the duodenal microbial N supply (g/ d) was estimated from the equation derived by Chen and Gomes (1992).

4.3.8 Statistical analysis

All statistical procedures were conducted using the software SAS 9.4 (SAS Institute Incorporated), having computed the arithmetic means per steer and period for all variables. For variables related to nutrient intakes, ATTD, performance, N use efficiency, rumen microbial protein synthesis, and blood metabolites, the data set consisted of 48 observations (i.e., 4 treatments × 3 periods × 4 steers). A mixed model (PROC GLIMMIX) was used for analysing all variables with the main effect of silage type, CP level, and their interactions (n = 16 steers) accordingly:

$$Y_{ijklm} = \mu + G_i + S_j + CP_k + T_l + (SCP)_{jk} + (SP)_{jl} + (CPP)_{kl} + (SCPP)_{jkl} + C_{mi} + e_{ijklm}$$

where Y_{ijklm} = dependent variable; μ = overall mean; G_i = effect of the steer group; S_j = effect of the silage type (j = 1-2); CP_k = effect of the CP level (k = 1-2); P_l = effect of the period (l = 1-3); $(SCP)_{jk}$ = interaction effect of the jth silage type and the kth CP level; $(SP)_{jl}$ = interaction effect of the jth silage type and the lth period; $(CPP)_{kl}$ = interaction effect of the kth CP level and the lth period; $(SCPP)_{jkl}$ = interaction effect of the jth silage type, the kth CP level, and the lth period; C_{mi} = random effect of the mth steer in the ith group; and e_{ijklm} = residual random error of experiment.

For methane data, 420 records were retrieved from each LMD measurement of the 4-min-time frame per steer, summing up to 241,920 records (420 records × 16 steers × 3 sampling days × 4 times measurement × 3 periods). The same mixed model as described for other variables above was used to analyse the methane data, including the RESIDUAL option of the RANDOM statement for the time factor. Least square means were declared at a significance level of $P < 0.05$.

4.4 Results

4.4.1 Chemical Composition of Diets

The concentrations of OM and ME were similar across all four diets, with an average (per kg DM) of 907 g, 8.8 MJ, respectively (Table 2). The aNDF, ADF and ADL concentrations of CS diet were reduced with high than low CP concentrations, whereas in CSS diets, the concentrations of aNDF, ADF, and ADL were greater with high than low CP concentrations.

4.4.2 Nutrient Intakes, Apparent Total Tract Digestibility, and Performance

Intakes of DOM and CP were lower in diets with low than high CP concentration ($P = 0.02$, $P < 0.01$; Table 3) for both silages, whereas CP intake differed across silages with greater CP intakes for corn-soybean than corn silage diets ($P < 0.01$). There was no interaction effect on nutrient intakes. Neither silage nor dietary CP and their interaction affected the intakes of DM, OM, aNDF, and ADF.

An interaction of silage type and CP level was observed for ATTD of CP ($P < 0.01$). As apparent digestion of CP decreased for high CP compared with low CP concentration for CSS diets, ATTD of CP was greater for high than low CP concentration for CS diets, with greater absolute differences between the CP levels in CS than CSS diets. Moreover, a tendency in the

interaction effect of silage type and CP level was found for the ATTD of DM, OM, and aNDF (P = 0.05). The ATTD of DM, OM and aNDF were greater for high than low CP concentration for both silages, with greater absolute differences between the CP levels in CS than CSS. The ATTD of ADF differed across dietary CP concentrations, with lower ATTD in diets with low than high CP concentration (P = 0.03). Besides, the ATTD of ADF was lower for CSS diets compared with corn silage diets (P < 0.01).

There was an interaction effect between silage type and CP level for the ADG and the feed use efficiency (P = 0.04 for both variables), with greater differences between CS and CSS at high CP than at low CP concentration. The initial LW differed between diets with either silage type, with greater initial live weight for CSS than CS (P < 0.01).

4.4.3 Purine Derivatives, Microbial Protein Synthesis, and Blood Metabolites

Neither silage nor dietary CP and their interaction affected the measured PD excretion. The urinary creatinine excretions only differed between both silage types, with greater creatinine excretion for CS than CSS diets (P = 0.02; Table 4). In addition, the ratio of total urinary PD excretions to urinary N excretion differed between silages and CP levels (P < 0.01 for both effects). The total PD to urinary N excretion ratio was lower for high than for low CP concentration and lower for CS compared to CSS diets. Neither silage nor dietary CP and their interaction affected ruminal microbial protein synthesis.

The BUN concentrations were lower for low than high CP concentration diets for both silages (P < 0.01). Besides, BUN concentrations were greater for CS than CSS diets (P < 0.01). Neither silage nor dietary CP and their interaction affected total protein in the blood.

4.4.4 Methane and Nitrogen Use Efficiency

An interaction effect between silage and CP level was found for the concentration of methane produced by growing steers (P = 0.03; Table 5). Methane concentrations were greater for high than for low CP concentrations of CS diets but greater for low than for high CP concentrations of CSS diets, with greater differences between CS and CSS diets at high than low CP concentrations.

In view of the differences in the CP concentration across diets, N intake differed with greater intake for high than low CP concentration (P < 0.01). In addition, N intake was greater for CSS than CS diets (P < 0.01). An effect of the interaction between silage type and CP level was observed for faecal N excretion (in g/d and g/100 g of N intake; P < 0.01 for both variables).

Faecal N excretions (g/d and g/100 g N intake) were lower for CSS than CS diets at low CP concentration and greater for CSS than CS diets at high CP concentration. For urinary N excretion (g/d), there were lower excretions for low than high CP concentration for both silages ($P < 0.01$), and N excretion (g/d) was greater in CS than CSS diets ($P < 0.01$). An effect of the interaction between silage type and CP level was observed for urinary N excretion (g/100 g of N intake; $P = 0.05$) with lower excretion for CSS than CS diets at low and high CP concentrations.

There was an interaction effect for the apparent retention of N irrespective of whether that was expressed per day, per 100 g of N intake, or per 100 g of digested N ($P < 0.01$ for all variables). Apparent N retentions (g/d, g/100 g N intake, and g/ 100 g digested N) were greater for CSS than CS diets at low CP concentration, whereas the differences between both silages at high CP concentration were not pronounced.

Table 3. Nutrient intake and apparent total tract nutrient digestibility of growing steers fed corn or corn-soybean silage at different dietary crude protein (CP; 115 and 135 g /kg dry matter (DM)) concentrations (least squares means, n = 12)

	Corn silage		Corn-soybean silage			*P*-value[b]		
Variable	CP-115	CP-135	CP-115	CP-135	SEM[a]	S	CP	S×CP
Intake (g/kg metabolic live weight)								
DM	22.8	23.5	23.5	22.9	0.06	0.86	0.88	0.08
Organic matter	73.8	76.3	75.4	74.8	0.63	0.99	0.57	0.35
Crude protein	9.70	10.9	10.5	11.3	0.08	<0.05	<0.01	0.36
Neutral detergent fibre	38.0	38.9	37.5	37.7	0.31	0.31	0.49	0.72
Acid detergent fibre	20.6	21.3	21.7	21.9	0.17	0.10	0.37	0.59
Metabolizable energy	72.7	73.3	71.9	72.6	5.93	0.64	0.68	0.99
Digestibility (g/kg)								
DM	626	666	620	625	13.4	0.01	0.01	0.05
Organic matter	577	615	573	583	12.2	0.01	<0.01	0.05
Crude protein	578	653	645	627	15.5	0.05	<0.01	<0.01
Neutral detergent fibre	493	544	485	490	19.1	<0.01	0.02	0.05
Acid detergent fibre	475	527	455	460	21.0	<0.01	0.03	0.06
Performance								
Initial live weight (kg)	169	171	172	172	6.50	<0.01	0.26	0.16
Final live weight (kg)	186	186	185	185	6.72	0.35	0.68	0.81
Average daily weight gain (kg/d)	0.79	0.64	0.58	0.58	0.06	<0.01	<0.05	<0.05
Feed use efficiency	0.16	0.13	0.12	0.12	0.01	<0.01	0.03	<0.05

[a] SEM, standard error of means

[b] Probability of an effect of silage type (S), crude protein (CP), and interaction effects of silage type with crude protein (S×CP).

Table 4. Purine derivatives (PD), ruminal microbial crude protein (MCP) synthesis, and blood metabolites in growing steers fed corn silage or corn-soybean silage at different dietary crude protein (CP; 115 and 135 g /kg dry matter (DM)) concentrations (least squares means, n = 12)

	Corn silage		Corn-soybean silage			*P*-value[c]		
Variable[a]	CP-115	CP-135	CP-115	CP-135	SEM[b]	S	CP	S×CP
Urinary PD excretion (mmol/d)								
Allantoin	58.1	54.0	56.3	55.3	4.59	0.93	0.38	0.59
Uric acid	5.38	7.51	5.42	5.93	0.57	0.03	<0.01	0.02
Total PD	63.4	61.6	61.6	61.3	4.66	0.74	0.73	0.80
Creatinine (mmol/d)	33.9	36.0	31.3	32.0	2.22	0.02	0.32	0.61
PD:creatinine index	105	94.6	97.5	93.6	7.04	0.38	0.14	0.48
Urinary PD: N ratio (mmol/g)	3.02	2.12	3.57	2.27	0.15	<0.01	<0.01	0.06
Urinary PD:creatinine ratio (mmol/mmol)	2.06	1.91	1.99	1.92	0.14	0.79	0.24	0.70
Ruminal MCP synthesis								
Microbial N flow (g N/d)	44.6	43.0	43.1	42.8	4.05	0.75	0.71	0.80
MCP (g N /100 g N intake)	55.9	48.6	50.7	48.2	5.07	0.31	0.07	0.38
MCP (g N /kg digestible OM intake)	30.1	25.1	29.1	28.5	3.75	0.65	0.31	0.41
Blood metabolites (mg/dL)								
BUN	9.77	13.5	6.86	10.8	0.45	<0.01	<0.01	0.61
Total protein	5.69	5.61	5.71	5.66	0.11	0.73	0.46	0.88

[a] N, nitrogen; BUN, blood urea-nitrogen

[b] SEM, standard error of means

[c] Probability of an effect of silage type (S), crude protein (CP), and interaction effects of silage type with crude protein (S×CP).

Table 5. Methane concentration, nitrogen (N) partitioning via urine and faeces, and apparent N retention in growing steers fed corn or corn-soybean silage at different dietary crude protein (CP; 115 and 135 g /kg dry matter (DM)) concentrations (least squares means, n = 12)

	Corn silage		Corn-soybean silage			*P*-value[b]		
Variable	CP-115	CP-135	CP-115	CP-135	SEM[a]	S	CP	S×CP
Methane (ppm)	33.2	36.7	35.7	31.1	2.02	0.07	0.93	0.03
N balance (g/d)								
N intake	80.9	92.1	89.1	94.5	4.76	<0.01	<0.01	0.07
Urine N	22.1	29.6	18.4	27.3	1.35	<0.01	<0.01	0.41
Fecal N	33.5	31.6	31.3	35.1	1.98	0.39	0.21	<0.01
Total N excretion	55.6	61.2	49.7	62.3	2.82	0.02	<0.01	<0.01
Fecal N: Urine N	1.63	1.11	1.79	1.30	0.09	<0.01	<0.01	0.79
N balance (g/100 g of N intake)								
Urine	27.5	32.5	20.9	29.3	1.19	<0.01	<0.01	0.05
Faeces	42.2	34.7	35.5	37.3	1.46	0.05	<0.01	<0.01
Apparent N retention								
(g/d)	25.3	30.9	39.4	32.4	2.73	<0.01	0.67	<0.01
(g/100 g N intake)	30.3	32.8	43.6	33.4	2.05	<0.01	<0.01	<0.01
(g/100 g digested N)	51.6	49.6	67.0	53.0	2.52	<0.01	<0.01	<0.01

[a] SEM, standard error of means

[b] Probability of an effect of silage type (S), crude protein (CP), and interaction effects of silage type with crude protein (S×CP).

4.5 Discussion

The two different silages in the present study were formulated to have two levels of dietary CP concentrations. Irrespective of these differences in CP levels, the proportion of silage and star grass hay in the diets was constant, while the ingredient composition of the concentrate proportion differed between CP levels and types of silages. Actual CP concentrations of the diets deviated from the targeted 115 or 135 g/kg DM for both CS and CSS diets. Nevertheless, absolute differences between low and high CP concentrations were similar for diets with both silage types. As a result of reducing dietary proportion for soybean meal and substituting it with ground corn in diets with low CP concentration, dietary starch concentration was numerically greatest for the low CP concentration diets and even lower in both CSS than the two CS diets. These differences in dietary starch concentrations should be considered when interpreting the observed treatment effects. Dietary aNDF, ADF, and ADL concentrations were similar across CP levels but differed between diets with both silage types, which may be responsible for differences in ATTD.

It was hypothesised that the effect of silage type would differ between CP levels, with feeding CSS at low CP concentration stimulating greater rumen MCP, improving N use efficiency and ADG compared to other diets. In contrast, CS fed at low CP concentration increased ADG more than other diets. However, the effects of CP level on several variables differed depending on the silage type. For instance, CP intake and ATTD of DM, OM, CP, and aNDF were lower with low than high CP concentration in both silage diets. Besides, the differences between CS and CSS were greater at high than low CP concentration for ATTD of DM, OM, and aNDF, except for ATTD of CP, where there were greater differences between CS and CSS at low than high CP concentration. Moreover, there were greater differences between CS and CSS at low than high CP concentrations for the efficiency of MCP synthesis (g/100 g N intake), N retention (g/d, g/100g N intake, and g/100g digested N), ADG, and feed use efficiency. The marked differences observed between CS and CSS diets at low than high CP concentrations in most of the studied variables may be related to the ratio of starch to protein supply to rumen microbes that was greater in low than high CP diets. In addition, changes in the microbial consortium of the rumen with different silages and CP levels could be anticipated, which

might explain the differences in nutrient fermentation and protein metabolism (Khafipour et al., 2016; Petri et al., 2013).

Given that nutrient ATTD depends on both cell wall concentration and its availability for digestion, the greater concentrations of ADF and ADL and the greater cross-linkage between lignin, hemicellulose and cellulose in the cell wall of CSS than CS diets might be responsible for the lower nutrient ATTD of CSS than CS diets. Contrarily, Souza et al. (2014) found no difference in the nutrient ATTD for CS in comparison with the increasing proportion of stylosanthes silage to replace CS in a TMR when fed to steers due to similar nutritional value of the two types of silage. The greater nutrient ATTD of high than low CP of both silage diets without a simultaneous increase in ADG and feed use efficiency of steers might indicate that postabsorptive losses of N were elevated with high CP diets (Arriola Apelo et al., 2014). The synchronisation of energy and protein supply for nutrient fermentation, which is not absolute (Merry et al., 2006), might elicit a greater positive response in nutrient ATTD for CS than CSS diets at high than low CP concentrations. It likely appears that the lower concentration of starch in CS diets at high CP concentration compensated for the greater nutrient ATTD than CSS diets due to a more favourable rumen condition for cellulolytic bacteria activity. Interestingly, the ATTD of CP that is usually greater to a certain degree with high than low CP concentration due to the dilution of metabolic faecal N and more digestible nutrients (Broderick, 2003) was greater with low than with high CP of CSS diets. The soybean forage component of CSS at low CP might have caused the greater digestion of CP due to higher post-ruminal digestion since the BUN concentration and urinary N excretions that reflect the ruminal CP degradation rate were lowest with CSS diet at low CP. Overall, it is suggestive that silage type affects nutrient supply.

Previous studies evaluating the effects of CP levels on steer performance have shown contrasting results. For instance, neither silage type nor CP levels affected the ADG and feed use efficiency of steers fed corn or stylosanthes silage at 110 or 130 g CP/kg DM in the study of da Silva et al. (2016), and this was due to similar total digestible nutrient intakes for steers. In contrast, Amaral et al. (2018) found an increase in the ADG and feed use efficiency of steers with an increase in CP levels when steers were fed a TMR with corn silage at dietary CP concentration of 100, 120, or 140 g CP/kg DM. Along the same line, an increase in ADG and feed use efficiency of steers in response to increased CP levels was observed across studies

(Bailey et al., 2008; Gleghorn et al., 2004; Rossi et al., 2001) when steers were fed a greater ratio of concentrate to roughage, suggesting that effects of dietary CP concentration are more pronounced than CP source. In the present study, steers' ADG and feed use efficiency improved when fed CS than CSS at low than high CP concentration, suggesting that the different diet composition might have affected the daily gain and feed use efficiency response. The net energy supply for gain was highest in the diet of CS at low CP compared to other diets, which might be responsible for the greater ADG and feed use efficiency in steers. Besides, the high variation in live weight measurement across steer groups and the relatively short periods associated with the experimental design of the present study, Latin square, might have impeded the estimation of ADG (Johnson et al., 2020). Similarly, changes in gut fill can influence the ADG significantly since changes in protein deposition were not measured exclusively (Schroeder and Titgemeyer, 2008).

The amount of nutrient supply and the availability of synchronised nutrients is vital to rumen MCP synthesis, which was not deficient in the present study. The compounding differences between silages and CP levels dictate the rate of energy and (or) protein supply in the rumen, with more preference for improved MCP efficiency for low CP concentration of both silage diets. Seemingly, the lower N intake and rumen degradable protein supply of low than high CP concentration in both silage diets was directed towards N incorporation into rumen microbial mass as it has been reported that at high N intake, the efficiency of conversion of N in silage based diets can be low (Givens and Rulquin, 2004). Likewise, the abundance of readily fermentable carbohydrates for both silages at low than high CP concentrations might have improved the MCP efficiency at low CP concentrations. At the same time, less energy was utilised for methane production for low CP concentration of CS than CSS in the present study, suggesting that the differences in the fibre and starch concentrations between CS and CSS diets might be responsible for the shift in energy utilisation. Additionally, the improved feed efficiency of CS might have reduced the methane emission intensity. Although the higher starch concentration in CSS than CS at low CP concentration may improve the capture of dietary N into MCP, there appeared to be a ceiling to the efficiency of the process overall (Lapierre and Lobley, 2001). Along this line, energy intake might have increased the efficiency of protein utilisation for CS than CSS and in low than high CP concentration diets as discovered for the greater ADG and feed use efficiency in CS than CSS diets. The marked

differences between CS and CSS at low CP in MCP efficiency are consistent with previous studies that showed legume and legume-grass silages typically have lower efficiency of MCP synthesis than grass or corn silages (Dewhurst et al., 2002, 2000).

Aside from nutrient fermentation and MCP synthesis, altering the rate and extent of fermentable energy and protein substrates in the rumen through nutrient supply also influence N use efficiency (Bach et al., 2005). The pronounced differences in the partitioning of N excretion between CP levels in both silages were expected and are in line with the results of previous studies (Broderick, 2018; Dijkstra et al., 2011; Hristov et al., 2019). Although faecal N excretion was greater than urinary N excretion in the present study, about 64% of dietary N ingested across diets was excreted via urine and faeces, and the environmental impacts can be undesirable. Increasing the dietary CP concentration, irrespective of silage reduced the concentration of fermentable carbohydrates. The BUN concentration is determined by carbohydrate and CP degradation and metabolism in the rumen. Hence, the surplus RDP supply that was not used for MCP synthesis was removed from the rumen pool by absorption through the rumen wall, leading to greater BUN concentration and urinary N excretion. The greater BUN concentration and urinary N excretion with high CP in both silage diets reflect the high degradability of the supplemental protein. This finding is consistent with previous studies that show a positive correlation between BUN and urinary N with the level of CP (Castillo et al., 2000; Dijkstra et al., 2011; Hristov et al., 2004; Lapierre and Lobley, 2001). Moreover, the lowest BUN concentration and urinary N excretion recorded for CSS diet with low CP indicate low degradation of CP in the rumen. The higher proportion of fibre-bound protein in CSS diets may be associated with the low CP degradation ruminally, thereby shifting the route of N excretion to faeces. In addition, the greater incorporation of dietary N into MCP with low than high CP, irrespective of silage, likely increased faecal excretion of microbial N. The increase in total apparent N retention for high CP of CS and low CP of CSS did not translate into a greater ADG of the growing steers in the present study. Apparent N retention was possibly overestimated due to the volatilisation of ammonia N in the pen during excreta collections. The lack of response indicates that other factors besides protein supply, for instance, differences in diet composition, affected protein use to promote ADG.

4.6 Conclusion

Increasing the CP concentration, irrespective of the silage type, did not improve animal performance and N retention but increased BUN concentrations and total N excretions, suggesting that dietary CP concentrations exceeded the requirement of rumen microbes in the growing tropical breed steers. Thus, CS with low CP concentration but a high fermentable energy supply supposedly improved nitrogen use efficiency with a higher microbial protein yield and animal performance than other diets.

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5 General discussion

5.1 Introduction

Silage production is as old as time because the beginning of this agricultural practice can be dated back to over 3000 years ago (Wilkinson et al., 2003). Besides hay production, silage making remains a viable option for farmers to conserve forages for an extended period commercially. Though silage making and its incorporation in ruminant feedstuff are well-established in Europe and North America, its adoption in tropical livestock farming has only become popular recently (Wilkins and Wilkinson, 2015). Primarily, the production of high-quality silage depends on controllable and uncontrollable factors, which differ between temperate and tropical zones (Bernardes et al., 2018). For instance, the climate-related factors of the tropical regions can adversely affect silage quality. Thus, the effect of feeding silages to ruminants cannot be isolated from the processes that occur during ensiling. It is crucial to continue to widen knowledge on silages in the tropics because it is gaining prominence in ruminant feeding.

It remains unclear why nutrient utilization of tropical legume silage in ruminants is more inconsistent than temperate legume silage, with dry matter intake and nutrient digestibility of tropical legume silage negatively affected (Castro-Montoya and Dickhoefer, 2018). One important factor could be that the effect of ensiling conditions was not considered when analyzing the nutritional variables from tropical legume silages. One of the main problems that usually occur during ensiling is the degradation of nutrients by microbes within the silo, particularly protein. This phenomenon increases at raised temperatures, leading to increased plant proteases activities, extensive hydrolysis of proteins, condensation of nutrients and increase in fibre-bound proteins (Dunière et al., 2013). Predominantly, the degradation of proteins by silage microbes is higher in forage legumes due to their high crude protein (CP) concentration and lower carbohydrates concentration than grasses prior to the first bite taken by ruminants (Foster et al., 2011). Consequently, another protein degradation occurs when the silage reaches the rumen, leading to significant protein losses at the animal level and reducing CP supply post ruminally. In view of the foregoing, supplementation of legume silage with another source of protein may be a valuable option in ruminant feeding. However, under different ensiling conditions, the nutritional changes that occur within silo may be amplified or reduced. The extent to which certain ensiling conditions, for instance, ensiling length and temperatures under natural conditions, will affect legume silage quality, and its utilization by

ruminant is still uncleared. Within this context was the present thesis designed to evaluate the effect of ensiling conditions on tropical forage legumes on a multi-level basis (i.e. silage level, rumen level, and animal level). As detailed discussions of a wide range of variables have been exercised in chapters 2, 3, and 4, in the following, rapt attention on specific results obtained in the different experiments of the study from silage production to animal performance will be discussed.

5.2 Effect of ensiling conditions on forage legumes in the tropics versus temperate

Production of high-quality silages depends on optimal ensiling conditions, and the silage fermentation pattern (extent and profile) further affects ensiled forage's nutritive value (Bernardes et al., 2018). However, a few studies considered the effect of ensiling conditions, such as ensiling length and temperature effect on ensiled forage legume compared to corn, and they are limited to the temperate. As a result, this section of the discussion will focus on comparing the result from the present study with a similar study conducted on the studied factor in the temperate regions of the world.

As revealed in the present study, the effect of ensiling length was more prevalent than storage temperature on all examined variables at the silage level. In Chapter 2, between days 0 and 75 of the ensiling length of silages of soybean, jack bean and lablab, dry matter (DM) and CP concentrations increased while the neutral detergent fibre (NDF) and acid detergent fibre (ADF) concentrations declined. Beyond 75 d of ensiling length, DM and CP concentrations reduced, whereas NDF and ADF concentrations increased. A similar study conducted by Santos and Kung (2016) on a low DM alfalfa at ensiling (similar to the DM at ensiling of the present study) found a decrease in the NDF and ADF concentration between days 0 and 180 and an increase in the NDF and ADF concentration from 180 to 360 days. Furthermore, the CP concentration increased with ensiling length from day 0 to 360 with no changes in DM concentration of alfalfa silages with increasing ensiling length. Although the time point of measurement of Santos and Kung study differs from the present study, it still appears that tropical forage legumes respond differently to prolonged ensiling length than temperate legumes, with a decline in the nutritive value after 75 days of ensiling. The stem to leaf ratio, the proportion of epidermis, bundle sheaths and vascular tissues are greater in forage legumes

of the tropics than temperate (Kering et al., 2011) which might have contributed to how tropical forage legumes behave in response to ensiling conditions. Previous studies have also demonstrated a close relationship between forage quality and plant tissue anatomy (Van Soest, 2018; Wilson, 1993). From a nutritional standpoint, much of the digestible nutrients conserved until 75 days of ensiling were used during silage fermentation leading to the rise in the fibre concentration of the legume silage after 75 days, thereby reducing the metabolizable energy (ME) concentration, an important indicator of silage quality. The decline in the lactic acid concentration of legume silage after 30 days of ensiling in the present study could be responsible for the loss of much of the digestible nutrients after 75 days. Contrariwise, the concentration of lactic acid increased with increasing ensiling length and was relatively stable for alfalfa in the study of Santos and Kung (2016). This difference indicates that the water-soluble carbohydrates of tropical forage legumes are lower than that of alfalfa.

5.3 Varying effect of ensiling conditions of sorghum-soybean on *in vitro* rumen fermentation and post-ruminal digestion of CP

The digestive interaction between the substrates contained within different forages, for instance, grass and legume mixtures, when fed to ruminants, can modulate the metabolic processes in the gastrointestinal tract (Niderkorn et al., 2011). Yet, the extent and profile of silage fermentation of mixed forages under various lengths of ensiling and storage temperature may affect the supply of CP and consequently the rumen fermentation profile and CP digestibility in ruminants. As revealed in Chapter 2 of the present study, the differing temperatures between outdoor and indoor storage affected the quality of CP of legume silages over time with the increase in the proportion of fibre-bound CP, especially when ensiled in combination with sorghum. This finding confirms that the increased Maillard reaction is a function of the length of exposure and the amplitude of temperatures (Guo et al., 2007; Muck et al., 2003). Thus, great concentrations of total CP do not necessarily relate to a greater nutritional value of silages because substantial CP proportions may be degraded in the rumen but not hydrolyzed in the intestine. Under these circumstances, the provision of additional CP sources may be a viable option to meet ruminants need and improve the utilization of the silage mixture.

Up until now, there were no studies to show the effects of ensiling length and temperature on *in vitro* rumen fermentation profile and post ruminal digestion of silages except for a few studies that examined the effect of ensiling length on ruminal *in vitro* starch digestibility of maize silage (Cone et al., 2008; Ferraretto et al., 2015; Gerlach et al., 2018). Chapter 3 of the present study showed a quadratic increase in the total gas production (GP), ammonia-nitrogen and total short-chain fatty acids (SCFA) concentrations of rumen inoculum with increasing ensiling length of sorghum-soybean silage (the greatest measure at 75 days of ensiling length). Considering that total GP and SCFA concentration are a predictor of carbohydrate degradation and ammonia-nitrogen concentration is an indicator of CP degradation in the rumen (Menke et al., 1979), it is suggestive that digestible nutrients conserved during ensiling were more at 75 d, thereby enhancing ruminal degradation. Besides, the higher GP and total SCFA of substrates from silages at 75 d compared to 0 and 180 d is associated with the substrates' low fibre concentration, indicating lower usage of readily fermentable carbohydrates by silage microbes during ensiling at 75 d compared to 180 d.

Regardless of the effect of ensiling lengths and storage temperature on sorghum-soybean proportion of the substrates (Chapter 3), the added CP source (soybean meal) enhanced the amount of CP in undegraded substrates and the apparent intestinal digestibility of CP. In contrast, the amount of CP in undegraded substrates and apparent intestinal digestibility of CP decreased linearly with increasing ensiling length (Chapter 3). Likewise, the apparent intestinal digestibility of CP for silages stored outdoors was lowered than those stored indoors. Typically, protein-rich ingredients like soybean meal are characterized by greater intestinal digestibility of rumen-undegraded CP as compared to forages (Westreicher-Kristen et al., 2018). The NRC (2001) model assumes that the intestinal digestibility of CP for soybean meal is 93% and 65% for all samples of forage legume silages. However, Boucher et al. (2009) study found an apparent intestinal CP digestibility of 98% for soybean meal using the modified 3-step procedure. Thus, it appears that the proportion of soy protein in the substrates with high CP concentration (Chapter 3) contributed significantly to the increase in CP of the undegraded substrates and the apparent intestinal CP digestibility compared to the substrates with low CP concentration. Besides, the possible overestimation of CP in undegraded substrates due to nitrogen contribution from rumen fluids might have contributed to the

increase in the proportion of CP in undegraded substrates and apparent intestinal digestibility of CP (Chapter 3). Although correction for microbial CP for substrates residues after the *in vitro* incubation to quantify the CP contribution of microbial origin was not done, the contribution of the silage CP proportion in the residue might be low, given that the acid detergent insoluble nitrogen (ADIN) and ADF concentration of sorghum and soybean silage are higher than that of soy protein. A recent study by Westreicher-Kristen et al. (2021) showed that exchanging the CP from soybean meal by CP from red clover silage in a total mixed ration (TMR) of dairy cow increased *in situ* ruminal CP degradation linearly but reduced the intestinal digestibility of the rumen-undegraded CP. The findings by Westreicher-Kristen et al. (2021) suggested that the increase in the lignin and ADF concentration of TMR by 46 % and 15% with an increase in the proportion of red clover silage might be responsible for the reduced intestinal digestibility of CP. Thus, the increase in the concentrations of ADF and ADIN with advancing ensiling length and in silages stored outdoors than indoors (Chapter 2) might have reduced the apparent intestinal digestibility of CP with increasing ensiling length and for silages stored outdoors and subjected to high ambient temperature (Chapter 3).

5.4 Complementarities between corn and soybean silage on nutrient utilization in ruminants

Beyond silage fermentation processes, there is the likelihood that the complementary effects between grass and legumes at ensiling may stimulate microbial CP (MCP) synthesis, thereby improving milk yield and weight gain when mixed silages are fed to ruminants (Auldist et al., 1999; Dewhurst et al., 2003). Nevertheless, the conditions at ensiling dictate the silage fermentation pattern (extent and profile), which may affect ensiled forage's nutritive value (Bernardes et al., 2018) and consequently nutrient utilization by ruminants.

Despite no difference in the nutrient intakes of corn and corn-soybean silage diet except CP intake that was greater in corn-soybean silage, the apparent total tract digestibility (ATTD) of all nutrients except CP was greater for corn than corn-soybean silage (Chapter 4). The greater ADF and acid detergent lignin (ADL) concentrations of corn-soybean than corn silage after 132 d of ensiling length is likely responsible for the reduced ATTD of nutrients. This assumption could be corroborated with similar findings in Chapter 2, which show a reduction

in ME and digestibility of organic matter (DOM) due to increased ADF and ADIN concentration of legume and sorghum-legume silages beyond 75 d of ensiling.

A closer look at the response to the ATTD of CP of the corn-soybean silage diet with the soybean proportion of the mixed silage as the primary CP source (low CP concentration, Chapter 4) shows the greatest ATTD of CP with the least total nitrogen excretion among the diets. The high ATTD of CP indicates the increased degradation of legume silage CP in the rumen, as confirmed in previous studies (Broderick, 2018; Givens and Rulquin, 2004). Although the total excretion of nitrogen for the corn-soybean diet (low CP concentration) was the least among all diets, it appears that enough CP from corn-soybean was incorporated for microbial growth. Besides, the amount of fermentable energy supplied in the diet was sufficient for synchronization with other ingredients predicated on the similarity in the MCP synthesis among diets.

Although the combination of corn and soybean at ensiling when fed to steers stimulated MCP synthesis and showed the greatest retained nitrogen in steers in the present study (Chapter 4), this finding did not translate into improvement in weight gain of steers compared with corn silage. Thus, it is suggestive that other factors like the proportion of other nutrients provided are essential factors in promoting CP supply for cattle performance (Clark et al., 1992).

5.5 Challenges of the present thesis

In Chapter 3, the undegraded CP in feed residues after *in vitro* incubation was greater than CP in the feed substrates because of additional nitrogen from microbial origin. Due to the small amounts of feed residues that were recovered and the small sample size, it was difficult to correct for the contributions of microbial CP in the undegraded feed residues as described by Edmunds et al. (2012). Hence, CP degradability was not reported, but the amount of CP in undegraded residues was reported. From this perspective, the apparent intestinal CP digestibility was overestimated, considering that the intestinal digestibility of microbial CP is 80% (Vérité et al., 1987).

For the *in vivo* study (Chapter 4), the initial plan was to ensile a mixture of whole cereal crop and forage legume under indoor and outdoor temperatures of the tropics for a prolonged period to alter the fibre-bound CP concentration before the feeding experiment. However, the

lack of silage baling machinery and other accessories that would have facilitated the easy storage of the ensiled forage in such ensiling conditions prevented the usage of this approach. Another approach to harvest forage legumes at different stages of maturity was considered to alter fibre-bound CP before ensiling with a whole cereal crop. Yet, rain flood negatively affected the emergence and yield of forage legumes planned to be harvested early. Ultimately, forage legumes harvested lately were combined with corn at ensiling and compared with whole corn crop silage during the feeding experiment. In the course of the *in vivo* study, total urine and faecal excretions were collected to estimate apparent nutrient digestibility and protein metabolism variables. However, minimal losses were recorded during urine collection from a few experimental steers that are unavoidable. In order to establish the precision of urine volume measurement in the study, the urine volume of each steer was estimated via the creatinine concentration method (Valadares et al., 1999). The data associated with the total collection of urine volume was compared with another set of data related to estimated urine volume via creatinine concentration. Nevertheless, the comparison showed that the associated data from the total urine collection obtained in the study was more plausible than data related to estimated urine volume via creatinine concentration.

5.6 Future perspectives

The production of high-quality silage is predicated upon optimal ensiling conditions. However, most silage studies in the tropical region focused on evaluating the impacts of varying pre-ensiling conditions on silage nutritional value with limited attention on how conditions during ensiling affect the nutritional value of silage and its utilization by ruminants. Based on the findings of this study, it is clear that even with the addition of silage inoculant, tropical forage legumes response to varying ensiling lengths was markedly different compared to a typical temperate forage legume (alfalfa). This difference is due to considerable breeding improvement in the water-soluble carbohydrate concentration of alfalfa cultivars over time, providing sufficient amounts of fermentable substrate for lactic acid production during prolonged ensiling. In the light of this, future breeding research would be essential in improving the non-structural carbohydrate concentration of atypical forage legumes in the tropics with similar qualities to temperates.

The degradation of proteins during ensiling of forage legume and when it reaches the rumen is inevitable. However, the presence of defensive mechanisms that protect protein in forage legumes, for instance, polyphenol oxidase and tannins, which form complexes that resist proteolysis, varies in quantity among forage legumes (Lee, 2014; Min et al., 2003). It is therefore essential to assess the protective activity of proteins by allelochemicals under varying ensiling length and different storage temperatures at silage and rumen level for tropical forage legumes with considerable amounts of allelochemicals. Additionally, microbiome studies at the silage level in view of storage temperatures effect would be essential to understand the activities of silage microbes.

As found in the present study, silages stored outdoor in the tropics had a higher concentration of fibre-bound protein than those stored indoor and consequently affected the intestinal digestibility of CP negatively using *in vitro* approach. Replication of such study with forage legumes ensiled in bales under indoors and outdoors temperatures of the tropics for feeding experiment in ruminants would give a clear understanding on the impacts of the changes in the protein fraction of legume silage at animal level.

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Chapter 6

6 Conclusions

The present thesis demonstrates that the utilization of nutrients of ensiled tropical forage legumes at ruminal and animal levels cannot be isolated from the processes that occur during ensiling, which is influenced by ensiling conditions. It becomes evident that the impact of ensiling length on the nutritional quality of tropical forage legume is overarching than storage temperature. Increasing the length of ensiling of forage legumes increased crude protein degradation, fibre concentration and condensation of fibre with crude protein. Thus, the impact of ensiling tropical forage legumes for a shorter period than a prolonged period on the nutritional quality of silage was positive through conservation of more nutrients at silage levels and the enhancement of nutrient utilization at ruminal and animal levels. In spite of the complementary effect of ensiling forage legumes and whole cereal crops together, prolonged ensiling under a characteristically high outdoor temperature of the tropics reduces the nutritional quality of silage and the nutrient utilization by cattle. Hence, supplementing prolonged silage mixture of whole cereal crop and forage legume with an additional source of crude protein and fermentable energy enhances nutrient utilization at ruminal and animal levels.