Nutritional Impact of *Desmanthus* as Protein Supplement on Tropical Beef Cattle Performance, Methane Emissions, Rumen Volatile Fatty Acids and Plasma Metabolite Profiles in Northern Australia

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Declaration on Ethics

The research presented and reported in this thesis was conducted according to the guidelines of the Australian Code for the Care and Use of Animals for Scientific Purposes (eight edition, 2013), and approved by the CSIRO Queensland Animal Ethics Committee (Permit Numbers A02/2018, 2019-32 and 2019-38).

Abstract

Beef production in northern Australia is characterised by an extensive grazing system dominated by tropical cattle breeds. Animal performance is generally low due to comparatively harsher climate, predominantly poor quality native pastures with low digestibility and higher methane (CH_4) emissions than the intensive system. *Desmanthus* is a tropically adapted legume that persists in a variety of heavy textured soils, heavy cracking clays, seasonally waterlogged duplex soils under low rainfall conditions with a promising potential for improving productivity in animals on poor quality diets. Very few *in vitro* studies suggested that *Desmanthus* could potentially mitigate CH_4 emissions, but to date, empirical evidence from *in vivo* feeding trial data to support this notion is lacking. Therefore, the series of studies reported in this thesis were designed to fill this significant knowledge gap with the objectives of exploring *in vivo* CH_4 mitigation capability of *Desmanthus* as a supplement in tropical beef cattle on poor quality feeds and the effects of its tannins and phenolic compounds on animal performance, rumen volatile fatty acids (VFA) and plasma metabolite profiles, relative to lucerne. The general hypothesis tested was that *feeding tropical steers with incremental levels of Desmanthus will linearly decrease CH_4 emissions, reduce proteolysis, lower rumen ammonia nitrogen and contribute to higher nitrogen flow without negatively impacting VFA, plasma metabolite profiles and liveweight (LW) gain*. Three *in vivo* experiments investigating the effect of supplementing tropical beef cattle with four different *Desmanthus* cultivars: JCU1 and JCU7 (*Desmanthus leptophyllus*), JCU4 (*Desmanthus bicornutus*) and JCU2 (*Desmanthus virgatus*) were conducted. Methane emissions were measured with open-circuit respiration chambers and GreenFeed emission monitoring systems. Results from the three experimental chapters demonstrated that while *Desmanthus* can prevent weight loss and marginally decrease CH_4 emissions in tropical steers feed poor quality diets, steers supplemented with *Desmanthus* recorded a lower dry matter intake and animal growth performance, but higher faecal nitrogen concentration than animals supplemented with lucerne. Among the three *Desmanthus* cultivars tested, there were no significant differences in nitrogen concentrations, VFA and plasma metabolite profiles. The addition of the tannin binder polyethylene glycol-4000 induced higher rumen iso-acid concentrations and faecal nitrogen excretion. It was concluded that on low quality basal diets, *Desmanthus* maintains LW, animal

health, improves nitrogen utilisation without negatively affecting rumen fermentation and plasma metabolite profiles. However, on high-quality diets, incremental levels of *Desmanthus* did not reduce CH_4 emissions, irrespective of the evaluation technique, as the ability of its tannins to mitigate CH_4 emissions was not evident. The thesis suggested areas for future studies.

List of Publications from Thesis

Peer-reviewed Journal Papers with 2020 Impact Factor (IF)

1. **Suybeng B**, Mwangi FW, McSweeney CS, Charmley E, Gardiner CP, Malau-Aduli BS, Malau-Aduli AEO 2021a. Response to Climate Change: Evaluation of Methane Emissions in Northern Australian Beef Cattle on a High Quality Diet Supplemented with Desmanthus Using Open-Circuit Respiration Chambers and GreenFeed Emission Monitoring Systems. *Biology* 10 (9): 943 **(IF 5.079**) DOI: https://doi.org/10.3390/biology10090943

2. **Suybeng B**, Charmley E, Gardiner CP, Malau-Aduli BS, Malau-Aduli, AEO 2021b. Plasma metabolites, productive performance and rumen volatile fatty acid profiles of northern Australian *Bos indicus* steers supplemented with *Desmanthus* and lucerne. *Metabolites* 11 (6): 356 **(IF 4.932**) DOI: https://doi.org/10.3390/metabo11060356

3. **Suybeng B**, Charmley E, Gardiner CP, Malau-Aduli BS, Malau-Aduli AEO 2020.Supplementing Northern Australian beef cattle with *Desmanthus* tropical legume reduces *in vivo* methane emissions. *Animals* 10 (11): 2097 **(IF 2.752**) DOI: https://doi.org/10.3390/ani10112097

4. **Suybeng B**, Charmley E, Gardiner CP, Malau-Aduli BS, Malau-Aduli AEO 2019. Methane emissions and the use of *Desmanthus* for beef cattle production in northern Australia. *Animals* 9 (8): 542 **(IF 2.752**) DOI: https://doi.org/10.3390/ani9080542

Conference Papers

1. **Suybeng B**, Charmley E, Gardiner CP, Malau-Aduli BS, Malau-Aduli AEO 2019a. *Desmanthus: A tropical legume for mitigating methane emissions in northern Australian beef cattle.* Paper presented at the 7th GGAA – Greenhouse Gas and Animal Agriculture Conference, 4-8 August 2019; Iguassu Falls, Brazil
2. **Suybeng B**, Charmley E, Gardiner CP, Malau-Aduli BS, Malau-Aduli AEO 2019b. *Desmanthus: A tropical legume for reducing methane emissions in northern Australian beef*

cattle. Paper presented at the Northern Beef Research Update Conference, 19-22 August 2019; Brisbane, Australia

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List of Abbreviations

ADF = acid detergent fibre

BUN = blood urea nitrogen

CP = crude protein

CR = clearance rate

CSIRO = Commonwealth Scientific and Industrial Research Organisation

CT = condensed tannins

DM = dry matter

DMD = dry matter digestibility

DMI = dry matter intake

DOMI = dry organic matter intake

FCR = feed conversion ratio

f.NIRS = faecal near-infrared spectroscopy

GEM = GreenFeed emission monitor

GHG = greenhouse gas

HT = hydrolysable tannins

JCU = James Cook University

LW = liveweight

ME = metabolizable energy

MLA = Meat & Livestock Australia

NDF = neutral detergent fibre

NEFA = non-esterified fatty acids

NIRS = near-infrared spectroscopy

OC = open-circuit respiration chamber

OM = organic matter

PEG = polyethylene glycol

REML = restricted maximum likelihood

RFI = residual feed intake

RFID = radio-frequency identification

RMSE = root mean square error

SEM = standard error mean

TP = total phenolics

VFA = volatile fatty acids

Chapter 1 : General Introduction

The world population is predicted to increase from 7.7 to 9.7 billion by 2050 (United Nations, 2019). Consequently, the world has to match the increased demand for food from a larger and more affluent population to its supply in an environmentally sustainable manner (Sarkar et al., 2020). Livestock products (meat, milk and eggs) constitute an important source of food for global food security by providing 31% and 15% of world protein and global per capita calorie consumption, respectively with regional variations (Godde et al., 2021). However, climate change constitutes a risk to livestock production due to its impact on the feed quality of crops and forages, animal performance, milk production, water availability, animal reproduction, livestock diseases and biodiversity (Sarkar et al., 2020). Global climate change is principally caused by greenhouse gas (GHG) emissions that result in warming of the atmosphere (Ridoutt, 2021). According to the Australian Greenhouse Emissions Information System (2018), 14.1% of GHG emissions emanate from agriculture, with methane (CH_4) accounting for 77.2% of total agricultural emissions. In 2018, Australia produced an estimated 51,668.30 Gg CO_2-e of CH_4 from enteric fermentation (Australian Greenhouse Emissions Information System, 2018). Therefore, the challenge is to find ways to increase livestock productivity without compromising household food security while sustainably improving the natural resource base (Panchasara et al., 2021).

Australia currently aims to reduce its carbon emissions by 26-28% below 2005 emissions by 2030 (Panchasara et al., 2021). In northern Australia (i.e. Queensland, Northern Territory and Western Australia above the Tropic of Capricorn), the beef industry is characterised by large pastoral properties with a herd population of about 14 million beef cattle. Pasture production is highly seasonal with grass growth occurring during the wet season (November to March), followed by a senescent period in the dry season. This induces a marked seasonal pattern of pasture availability and quality (Schatz et al., 2020). The dominant pasture species are C4 grasses, which generally have lower nutritional value than temperate grasses and are characterised by lower individual animal performance than grasses in temperate regions (Bowen et al., 2018; Whitley et al., 2017). According to Johnson and Johnson (1995), low animal productivity is associated with high methane output per unit of product and low pasture

quality is associated with high methane output per unit of dry matter intake (DMI). Thus, CH_4 emissions from the northern Australian beef herd are considered to be higher than for more intensive systems and are responsible for about 5% of the nation's total GHG emissions (Durmic et al., 2017).

In northern Australia, the grazed area is over 145 million hectares including in majority native pasture (primarily Mitchell grass (*Astrebla* spp.)), forbs, shrubs and 36% of sown pasture (primarily buffel grass (*Cenchrus ciliaris*)) (Bowen and Chudleigh, 2020; Costa et al., 2012). The region is characterised predominantly by a tropical and arid climate with a rainfall occurring mainly over summer with an annual average rainfall of 550-650 mm (Brown et al., 2019). Daily mean temperatures are comprising between 27 °C and 33 °C with the highest temperatures experienced in January (up to 36 °C). In winter, temperatures are around 18-27 °C with the lowest temperatures happening in July (down to 12 °C) (Moise et al., 2015). The property size is averaging 50,000 ha with a stocking rate of around one animal to every 10 ha (Bowen and Chudleigh, 2020). The properties in the region practice controlled or continuous mating with two annual musters in order to wean calves and recognize breeding cows for culling. Heifers are separated from the breeding herd until first mated at the age of two. The steer liveweight gain is about 130 kg/head/annum with a turn-off of 2-3.5 years at a liveweight of around 400 kg (Bowen and Chudleigh, 2020). Some approaches are adopted by producers in northern Australia to reach higher priced markets such as moving weaners to either higher quality grazing land situated in central and southern Queensland or to backgrounding pasture based systems prior going to a feedlot usually located in southern Queensland or northern New South Wales (Costa et al., 2012). Some producers also export live cattle mainly to Indonesia and Vietnam with 1.3 million head exported in 2019 (MLA, 2020).

Beef cattle productivity in north Queensland is beset with climatic and nutritional challenges due to prolonged drought, high climate variability, inadequate feed resources, low-quality pastures, low concentrations of phosphorus and nitrogen in soil, and poor body condition of cattle (Bowen and Chudleigh, 2019; Bowen et al., 2020; Bowen et al., 2021; Cobon et al., 2020). In heavy textured soils, dissolved organic nitrogen (N) constitutes about 40% of total N losses in deep drainage which induces a deep drainage of dissolved organic carbon. Both nitrate-N and dissolved organic carbon can cause

denitrification in deeper soil horizons (Nachimuthu et al., 2019). In this seasonally dry, low-elevation, heavy textured soils, inland tropical region of North Queensland, there is an overwhelming need for integrating more productive, nutritious and persistent summer-growing legumes into existing low quality, grass-dominant pastures. Beneficial outcomes to the livestock industry include improved body condition, higher growth rates of beef cattle, faster turn-off rate, improved heavier carcass weight, higher dressing percentage and a potential improvement in sensory meat eating quality traits (Coates et al., 1997; Vasta et al., 2007; Winks, 1973). For instance, Bowen et al. (2018) reported a 1.6 and 2.6 times the annual cattle liveweight (LW) gain per ha for animals grazing a butterfly pea (*Clitoria ternatea*) + perennial C4 grasses and Leucaena (*Leucaena leucocephala* spp. *glabrata*) + perennial C4 grasses respectively compared to perennial grass pastures only.

Suitable sown legumes are not available for all environments because existing cultivars lack the ability to adapt to infertile light-textured soils, heavy cracking clays, seasonally waterlogged duplex soils and low rainfall conditions (Hall and Walker, 2005). Even where soils are fertile and support productivity in introduced legumes (*Chamaecrista rotundifolia* and *Stylosanthes* spp.), the variable and low rainfall limits legume performance (Boschma et al., 2021). Constraints to the use of sown tropical pastures include limited availability of well adapted legumes to clay soils, instability of legume-based pastures and the high cost of establishing and maintaining improved pastures (Burt and Lazier, 2016; Burt et al., 2016). Selection of environmentally well-adapted and vigorous legumes with an ability to spread under grazing in the dry tropics will help alleviate these constraints. Gardiner (2016) evaluated the performance characteristics of *Desmanthus (D. bicornutus, D. leptophyllus* and *D. virgatus)* in contrasting tropical environments and found that it thrived and spread on heavier vertisol soils. In a study of pasture legume adaptation to six environments in the seasonally dry tropics of North Queensland, Hall and Walker (2005) demonstrated over a 15-year period, that on cracking clay soils, *Desmanthus* species and *Clitoria ternatea* were the most productive and persistent legumes among 118 legumes accessions.

Desmanthus cultivars have also demonstrated an anti-methanogenic potential *in vitro*. Vandermeulen et al. (2018) demonstrated a potential reduction of CH_4 emissions by up to 50% compared to Rhodes

grass with *Desmanthus leptophyllus* cv. JCU 1 after 51 days regrowth using rumen fluid from Brahman steers. Durmic et al. (2017) reported a potential 48% *in vitro* mitigation in summer with cv. JCU 1 using sheep rumen fluid compared to the average CH_4 emissions of 23 tropical grasses. Vandermeulen et al. (2018) attributed the reduction of CH_4 emissions to the presence of secondary compounds such as hydrolysable tannins (HT), condensed tannins (CT) and/or their combination in *Desmanthus* spp. Plant secondary compounds such as phenolics which include CT and HT, have an important role in feeding strategies to mitigate CH_4 emissions from ruminants (Aboagye and Beauchemin, 2019; Terranova et al., 2020). Tannins, which are polyphenolic molecules, have the ability to complex with proteins and to a lesser extent, with metal ions, amino acids and polysaccharides (Makkar, 2003b). This aptitude enables a decrease in rumen degradability of crude protein (CP) and sometimes CP digestibility in the digestive tract, which shifts N loss from urine to faeces (Grainger et al., 2009; Lagrange et al., 2020; Tseu et al., 2020). Nitrogen plays a crucial role in the development and growth of animals. Approximately 70% of the nitrogen ingested is excreted in the faeces and urine, which induces environmental pollution and limits animal productive performance (Yang et al., 2021). Tannins have also been described as anti-nutritional factors because of their negative effect on animal nutrition (lower feed intake, dry matter digestibility (DMD) and LW gains) (Mueller-Harvey, 2006). Furthermore, previous studies showed significant LW gains in steers (Collins et al., 2016; Gardiner and Parker, 2012), sheep (Ngo, 2017; Rangel and Gardiner, 2009), and goats (Aoetpah et al., 2018) supplemented with *Desmanthus*. *Desmanthus* has the potential to be a promising legume for animal growth and CH_4 reduction. However, to the researcher's current knowledge, studies exploring *in vivo* CH_4 mitigation capability of *Desmanthus* as a supplement in tropical beef cattle on poor quality feeds and the effects of tannins and phenolic compounds in *Desmanthus* on animal performance and CH_4 emissions are either very scanty or lacking.

During growth and development, cattle go through physiological and metabolic adjustments which can be monitored by specific blood metabolome that provide a suite of predictive biomarkers for livestock health, performance and disease (Connolly et al., 2020; Goldansaz et al., 2017). For instance, high energy content in the diet triggers carbohydrate fermentation and results in an increase in rumen volatile

fatty acids (VFA) that are absorbed and converted into glucose as energetic compounds for the body (Richards et al., 1995). On the other hand, lack of sufficient energy in feeds leads to mobilisation of body reserves including body proteins, which in turn, elevates urea concentration in the blood (Greenwood et al., 2002). However, to the researcher's current knowledge, there are no existing peer reviewed reports on the plasma metabolite profiles of tropical northern Australian beef cattle steers supplemented with *Desmanthus*.

Open-circuit respiration chambers (OC) have been considered as the "gold standard" to precisely measure CH_4 emissions from rumen and hindgut fermentation (Thompson and Rowntree, 2020). However, OC can be intrusive for the animals inducing a reduced feed intake and thus a higher CH_4 yield (expressed as g/kg DMI) (Llonch et al., 2016; Llonch et al., 2018). The GreenFeed emission monitoring (GEM) system which is a patented automated head-chamber system has shown minor difference for average values of CH_4 emissions compared with OC (Doreau et al., 2018). The GEM technique has the advantage of reducing labour input and interference with animal behaviour and production (Waghorn et al., 2016). Consequently, GEM can be used in grazing situations (Huhtanen et al., 2019). However, no *in vivo* study has been conducted using OC or GEM to measure CH_4 emissions from beef cattle supplemented with *Desmanthus*.

The series of studies reported in this thesis are based on *in vivo* experimentation with beef cattle under tropical management practices with the over-arching objectives of investigating:

1) Exploration of literature to identify current knowledge gaps in *in vivo* CH_4 emissions research;
2) *In vivo* CH_4 emissions, feed intake, LW gain, N utilisation, plasma and rumen metabolite responses of tropical beef cattle to incremental levels of supplementation with *Desmanthus* cultivars JCU1 *(D. leptophyllus),* JCU2 *(D. virgatus),* JCU4 *(D. bicornutus)* and JCU7 *(D. leptophyllus);*
3) Effect of tannin and phenolic components in *Desmanthus* (same cultivars as above) on CH_4 emissions, feed intake, N utilisation, rumen VFA and plasma metabolite profiles;
4) Evaluation of CH_4 emissions using OC and GEM systems.

These objectives were established to provide tropical beef cattle farmers in northern Australia with evidence-based and scientific data-driven information about the potential of *Desmanthus* to reduce CH_4 emissions, improve animal growth and N utilisation, and to investigate its effects on rumen fermentation and plasma metabolites. Therefore, this thesis is structured into the following chapters:

Chapter 1: General Introduction

Chapter 2: Literature Review: An in-depth and systematic exploration of the published literature on current contextual background of Australia's tropical beef cattle production system with emphasis on the use of *Desmanthus*, a tropical legume, as a nutritional supplementation strategy for the mitigation of CH_4 emissions and improvement of animal growth performance. It also identifies current knowledge gaps in *in vivo* CH_4 emissions research.

The successive chapters are investigative experimental studies that describe the effects of dietary supplementation with *Desmanthus* on feed intake, CH_4 emissions, growth, rumen VFA, plasma metabolites and N utilisation in tropical beef cattle steers.

Chapter 3: The main objective of this chapter was to investigate the effects of supplementing beef cattle with incremental levels of *Desmanthus leptophyllus* cv. JCU1 and *D. bicornutus* cv. JCU4 (which showed a higher anti-methanogenic potential compared to JCU2 *in vitro*) on *in vivo* CH_4 emissions, dry matter intake, LW gain and plasma metabolites. It also aimed to study the tannin effect on CH_4 emissions, intake, LW gain and rumen metabolites by adding the tannin binder polyethylene glycol-4000 (PEG). The hypothesis tested was that *feeding tropically adapted steers with incremental levels of JCU1 and JCU4 will linearly decrease CH_4 emissions due to the presence of tannins without negatively impacting rumen VFA, plasma metabolite profiles and LW gain*.

Chapter 4: The objective of this chapter was to compare animal productive performance, N dynamics, VFA and plasma metabolite profiles in tropical steers offered *Desmanthus virgatus* cv. JCU2, *D. bicornutus* cv. JCU4 and *D. leptophyllus* cv. JCU7, relative to lucerne, a temperate legume widely characterised for its high nutritive value. The chapter also investigated the role of condensed tannins and total phenolics (TP) in the utilisation of N in *Desmanthus* relative to lucerne, with the addition of

PEG. This chapter tested the hypothesis that *the presence of Desmanthus and thus tannins, will reduce proteolysis in the rumen, lower rumen NH_3-N and contribute to higher N flow to the lower tract resulting in higher faecal N concentration*.

Chapter 5: The first objective of this chapter was to compare the effect of supplementing tropical beef cattle with JCU2, JCU4 and JCU7 or lucerne on *in vivo* CH_4 emissions measured by open-circuit respiration chambers. The second objective was to investigate the effect of incrementally supplementing tropical beef cattle with an equal proportion of the three *Desmanthus* cultivars on CH_4 emissions measured by the GreenFeed emission monitoring system. It also investigated the effect of tannins on CH_4 emissions with the addition of PEG. The chapter tested the hypothesis that *increasing the proportion of Desmanthus in the diet will reduce CH_4 emissions when measured by GEM*.

Chapter 6: This chapter is a general discussion of the main thesis outcomes.

Chapter 7: This chapter is the conclusion and highlights areas warranting further investigation.

Appendices: Contains all supplementary materials and copies of peer-reviewed publications from this thesis.

Chapter 2 : Literature Review - Methane Emissions and the Use of *Desmanthus* in Beef Cattle Production in Northern Australia

2.1. Carbon Footprint from the Beef Industry in Queensland

2.1.1. The Australian Beef Cattle Market

Australia is the second biggest beef and veal exporter in the world with 1,750,000 tons of carcass weight exported annually. The Australian off-farm meat value (domestic expenditure plus export value, including live export) of the beef industry accounted for $20.2 billion in 2019 (MLA, 2020). Furthermore, the beef cattle industry employed 189,000 people in 2019 (MLA, 2020). Therefore, the beef industry plays a central role in the Australian economy, especially in the state of Queensland, where its 11.3 million head of cattle accounted for 47% of the Australian beef and veal production in 2019 (MLA, 2020).

2.1.2 The Different Sectors Included in the Carbon Footprint of the Beef Industry in Queensland

The total net emissions attributed to agriculture in Queensland was 21,173.16 Gg CO_2-e in 2018 (Australian Greenhouse Emissions Information System, 2018). The beef industry in Queensland is the largest agricultural industry in the state (Bray and Willcocks, 2009). Sources of GHG emissions from a typical beef enterprise comprise enteric fermentation in cattle (CH_4 and N_2O), burning of vegetation (intentional or accidental), energy use (electricity and fuel), land clearing, loss of pasture and decline in soil carbon (Bray and Willcocks, 2009; Eady et al., 2011). A study conducted by Eady et al. (2011) in two beef farms in Queensland showed that the carbon footprint of beef products at the farm gate ranged from 17.5–22.9 kg CO_2-e/kg liveweight at Gympie and 11.6–15.5 kg CO_2-e/kg liveweight in the Arcadia Valley. They also found that enteric fermentation represented about 80% (74% at Arcadia Valley and 85% at Gympie) of the overall 'cradle-to-farm gate' GHG emissions. The last figures can be linked with the 75% (15,960.67 Gg CO_2-e) of agriculture GHG emissions coming from enteric fermentation from grazing beef cattle in Queensland in 2018 (Australian Greenhouse Emissions Information System, 2018).

2.1.3. The Principal Causes Inducing Enteric Methane Emissions

2.1.3.1. Rumen Microbial Fermentation

The rumen is a dynamic and complex ecosystem composed essentially of anaerobic bacteria, protozoa, anaerobic fungi, methanogenic archaea and phages (Morgavi et al., 2010). The microbes interact with each other and have a symbiotic relationship with the host. The breakdown of plant cell wall carbohydrates that are inedible by humans provides energy to the host (Huws et al., 2018). Methane is produced exclusively by methanogenic archaea (Morgavi et al., 2010) via the hydrogenotrophic pathway using CO_2 as the carbon source and H_2 as the main electron donor, and less so through the utilisation of methyl groups (methylotrophic pathway), or even less commonly from acetate (acetoclastic pathway) (Morgavi et al., 2010). The methanogenesis reaction uses H_2 to reduce CO_2 to CH_4: $CO_2 + 4H_2 = CH_4 + 2H_2O$ (Thampi et al., 1987).

The main products of rumen microbial fermentation, as depicted in Figure 2.1, are volatile fatty acids (VFA) (acetic, propionic and butyric acids), carbon dioxide and methane (Immig, 1996). In the rumen, the VFA formed are absorbed and used as a source of energy. On the contrary, CO_2 and CH_4 are eliminated by eructation from the rumen. Over 80% of the methane is synthesised in the rumen and the lower digestive tract produces the rest (Immig, 1996). Northern beef cattle in Australia can generate about 32.2 to 184 g of methane per day (Charmley et al., 2016), which represents an important energy loss to the animal ranging from 2% to 12% of gross energy intake depending on the nature of the diet (Johnson and Johnson, 1995). Under a high forage diet, these losses are on the average, 7.2% of gross energy intake; 6.3% for an intermediate forage and 3.84% for a low forage (feedlot) (Gavrilova et al., 2019).

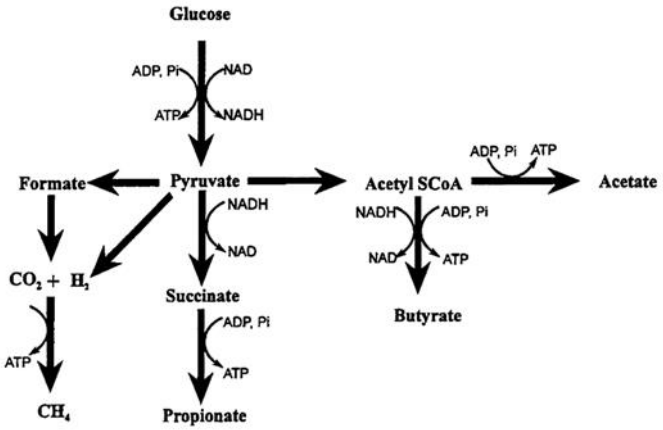


Figure 2.1. Principal end products of carbohydrate fermentation in the rumen (Immig, 1996)

2.1.3.2. Low Animal Performance Increases Methane Production

Less efficient cattle can take longer to reach market weight and might only breed two out of three seasons. The longer an animal takes to reach market weight, the longer that animal is producing methane, with very little beef being marketed in return (Charmley et al., 2008; Johnson and Johnson, 1995). Arthur et al. (2001) estimated genetic and phenotypic parameters for feed intake in Angus bulls and heifers, and showed that the feed conversion ratio defined by the amount of feed consumed divided by LW gain was correlated genetically (−0.62) and phenotypically (−0.74) with the daily LW gain. For instance, Charmley et al. (2008) showed that by maintaining a LW gain of 0.5 kg/day for steers in the northern spear grass region by adding supplements to the pasture diet would reduce the turn-off age of the Japanese Ox market from 4 years (526 kg LW) to 2.3 years (650 kg LW). Gross margin budget and cashflow analyses for a 100-cow herd showed a 61% internal rate of return over a 25-year investment period, despite the higher cost for purchasing efficient bulls. It represents an annual benefit per cow of A$8.76 (Exton et al., 2000). Low animal productivity is associated with high methane output per unit of product (methane intensity) and low pasture quality is associated with high methane output per unit of dry matter intake (DMI) (Archimède et al., 2011; Beauchemin et al., 2008). For that reason, Northern Australian beef herds are estimated to produce more methane than the more intensive systems in Southern Australia (Johnson and Johnson, 1995). For instance, Eady (2011) showed that the GHG emissions of beef produced from cattle supply chain from northern Australia to the Indonesian market were higher (26 kg CO_2 equivalent/kg liveweight) than beef produced in Southern Australian systems,

where GHG emissions ranged from 5.4 to 14.5 kg CO_2 equivalent/kg liveweight for finished steers. They attributed it to the higher reproduction rate, faster turn-off and lower methane emissions per unit of feed intake permitted by a high pasture quality in the southern system (Eady, 2011).

2.1.3.3. Northern Australian Forage Diet Influences Rumen Microbiome and Methane Production

In northern Australia, comprising the Kimberley and Pilbara districts of Western Australia, the Northern Territory and Queensland above the Tropic of Capricorn, the beef industry is dominated by large pastoral properties (Gardiner, 2016). This part of Australia is characterised by a vast array of heavy clay or vertisol soils, where the range of available sown pasture legumes has long been regarded as being deficient (Pengelly and Conway, 2000). There are also vast areas of light textured soils where the legume *Stylosanthes* has been successfully introduced. Pasture production is highly seasonal, with a wet season (November to April) characterised by growth, and a senescent period during the dry season. This induces a marked seasonal pattern of pasture availability and quality (Tothill and Gillies, 1992). The prevailing pasture species are mainly C4 grasses, which have lower nutritional value than temperate grasses, and result in lower animal productivity than in temperate regions (Hattersley, 1983; Perry et al., 2017). During the wet, hot summers, these pastures grow quickly and persist through the dry winter seasons as mature grasses (Hennessy et al., 1983; McLennan, 1997; Shaw and Bisset, 1955). The low livestock productivity in northern Australia is especially due to low protein content and low digestibility during the dry season (Poppi and McLennan, 2010). The low digestibility (45% organic matter) and nitrogen content (less than 7g N/kg dry matter (DM)) of these grasses during the dry season results in poor forage intakes and low annual growth rate of young cattle (Hennessy et al., 1983; McLennan, 1997; Shaw and Bisset, 1955). Animals tend to put on weight in the wet season and lose weight in the dry season. In northern Australia, it is not uncommon for 4–6 years old steers to be marketed (Poppi and McLennan, 1995). Consequently, depending on the time of the year, LW gains in northern Australia are around 70–240 kg/year for native pastures (Bortolussi et al., 2005) compared to 250–300 kg/year for temperate pastures (Poppi and McLennan, 2007). Growth rate is directly related to metabolizable

energy intake, and can be markedly increased by replacing the feed base or by giving supplements to the animals (Poppi and McLennan, 2007).

Archimède et al. (2011) showed that ruminants fed C4 grass produced 17% more methane as L/kg organic matter intake than those fed C3 grass. Likewise, Perry et al. (2017) found that steers fed a wet season pasture (CP = 90 g/kg DM) or a high quality hay (CP = 88 g/kg DM) produced 5–10 g CH_4/kg, digested less DMI and had about 3% less digestible energy intake than steers fed low quality hay (CP = 25 g/kg DM). They observed shorter rumen retention times in high quality hay fed steers, which decreased methane production per kilogram of DMI compared with low quality hay and the dry season pasture. This phenomenon can be explained with an increased rumen outflow rate (Benchaar et al., 2001). The rise in rumen outflow rates is associated with higher concentrations of dissolved H_2 that increase the growth rate of methanogens. The greater cellulose and hemicellulose content in tropical C4 grasses rather than neutral detergent soluble carbohydrates in grain diets results in higher methane emissions and a shift in rumen fermentation pathways from propionate to acetate (Perry et al., 2017). The production of methane in the rumen is associated with the production of VFA. The formation of both acetic and butyric acids is accompanied by the production of H_2 and CO_2, whereas propionic acid production requires a net uptake of H_2, which can reduce methanogenesis (Benchaar et al., 2001). The production of propionic acid instead of acetic acid can be realised by replacing structural carbohydrates (forage) with easily fermented carbohydrates (Benchaar et al., 2001).

2.2. Methane Measurement Techniques

The accurate measurement of methane emissions from ruminants is necessary for developing a robust inventory and mitigation strategies for the environmental footprint from animal production systems and enable the generation of carbon credits (Goopy et al., 2016; Zhao et al., 2020b). Methane emissions can be affected by production efficiency, behaviour, growth stage, dietary chemical composition, and animal management. As such, any methods impacting these factors will limit its usage for methane measurement (Zhao et al., 2020b). *In vitro* incubation, *in vivo* indoors (open-circuit respiration chambers, sniffer method, ventilated hood) or outdoors (GreenFeed, sulphur hexafluoride tracer, laser methane detector) techniques have been used to measure methane emissions from ruminants (Zhao et al., 2020b).

2.2.1. Indirect Methane Emissions Estimation – the In Vitro Incubation Technique

The *in vitro* technique principle is based on the incubation of rumen inoculum with a feed substrate under an anaerobic environment in gas-tight culture bottles. The gas emitted is recorded over time. The gas production values are corrected for the amount of gas produced in a blank incubation and these values can be fitted with time using a nonlinear curve fitting procedure in GenStat or other suitable statistical analysis software (Goopy et al., 2016). Methane concentration is usually determined by gas chromatography (Kinley et al., 2016). Yanez-Ruiz et al. (2016) reviewed 10 studies that compared *in vitro* and *in vivo* methane emissions. When methane emissions are expressed per unit of degraded rather than ingested material, both *in vitro* and *in vivo* methane production are closely correlated. Therefore, *in vitro* testing of anti-methanogenic additives is valuable prior to *in vivo* testing. They recommend that *in vitro* data are confirmed *in vivo* before making any conclusions on the effectiveness of supplements for lowering methane production because inhibition potential is often over-estimated *in vitro.*

2.2.2. Direct Methane Emissions Measurement

2.2.2.1. Open-circuit Respiration Chambers

Methane chambers have often been considered as the "gold standard" to accurately measure methane production from rumen and hindgut fermentation (Charmley et al., 2011; Goopy et al., 2016; Thompson and Rowntree, 2020; Zhao et al., 2020b). Although chamber designs vary, the basic principle remains the same. Sealed and environmentally controlled chambers are constructed to house test animals. A detailed description of the design and construction of low-cost open-circuit respiration methane chambers has been described by Klein and Wright (2006). Briefly, the chambers are constructed from square aluminium tubing and covered by thick, UV-resistant, clear and flexible polyvinyl chloride sheeting for the animals to be able to see each other (Williams et al., 2007). Methane emissions are analysed using an infrared gas analyser and calculated by multiplying the measured airflow by the difference in concentration between the inlet and outlet air (Williams et al., 2007) (Figure 2.2). Often, a multi-gas analyser which measures CH_4, CO_2, H_2, O_2 and NH_3, is simultaneously used (Klein and Wright, 2006; Martinez-Fernandez et al., 2016). Respiration chambers have numerous advantages:

Methane emissions can be recorded within a few minutes, but also for 24 h a day for a few days to estimate the mean daily methane production (Charmley et al., 2016). Measurements for different species of animals can be done depending on the size of the chambers; as exemplified in dairy cows (Denninger et al., 2020), steers (Charmley et al., 2016), goats (Abecia et al., 2012), sheep (du Toit et al., 2020) and red deer (Pérez-Barbería et al., 2020)). However, methane chambers are expensive to construct, maintain and technically demanding to operate. Moreover, respiration chambers cannot be used to measure many animals at once (Zhao et al., 2020b). The animals also require training (Williams et al., 2007), feed intakes are generally lower in respiration chambers and could possibly induce a higher methane yield measurement (Llonch et al., 2016).

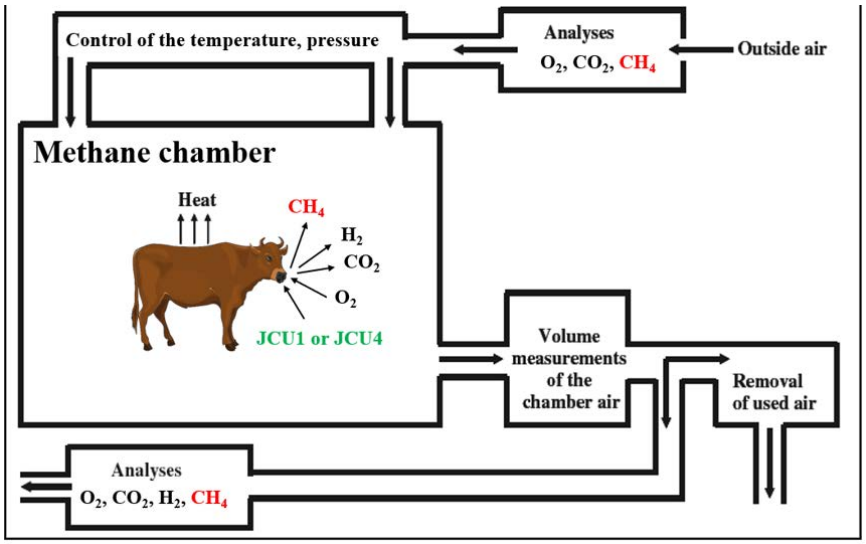


Figure 2.2. Design of the respiration chambers. Adapted from Makkar and Vercoe (2007)

Another technique similar to the respiration chamber called ventilated hood or respiration head box, has been developed to simplify operations of the respiration chambers. It uses a similar gas measurement technique that covers only the head of the animal instead of its whole body (Place et al., 2011). Castelán Ortega et al. (2020) showed that the construction of this device was 70% cheaper than that of a conventional respiration chamber and provides results that are consistent with respiration chamber data reported in the literature. A critical limitation of the hood system is that extensive training is necessary to allow the animals to get used to the hood apparatus. Thus, it is unsuitable for measuring emissions by many animals (Goopy et al., 2016).

2.2.2.2. Greenfeed Emission Monitoring System

GreenFeed emission monitoring (GEM, C-Lock Inc, Rapid City, SD, USA) system is a patented automated head-chambers system based on spot sampling (2-6 min) of eructated and exhaled gases allowing measurements of enteric methane production on a large number of animals under on-farm conditions (Huhtanen et al., 2019). An active airflow is induced to capture emitted air (flux method) and measure methane emissions just as in the respiration chambers (Figure 2.3). This system integrates measurements of air flow, gas concentration and detection of muzzle position to allow direct measurement of CH_4 and CO_2 fluxes during each animal's visit to the feed trough (Huhtanen et al., 2015). The 24-hour machine's availability reduces potential sampling bias and the technique has been shown to give similar results as the respiration chambers and SF_6 techniques (Jonker et al., 2020). This technique can be implemented in grazing trials with minimal labour input and minimal interference with animal behaviour and production (Waghorn et al., 2016). Data are uploaded to a cloud-based analysis system in real-time developed by the GEM manufacturer for methane emission estimations (Hammond et al., 2015). However, this system requires provision of supplemental feeds in the unit to entice the animals. Supplemental feeds may be a concern in animal nutrition studies due to the possibility of an excessive contribution of enticement feed to the diet, although restrictions are imposed. Moreover, multiple animals using the unit can alter the temporal distribution of measurements for individual animals (Goopy et al., 2016; Hammond et al., 2015). The methane measurements being different depending on the time of the day and between the individual animals (Hammond et al., 2016), it has been recommended to strictly adhere to the calibration and background collection from Hristov et al. (2015). Furthermore, as it is voluntary for the animals to be measured, it could limit the measurement timing, frequency of individual animals and unbalance the number of animals measured in different treatment groups (Hammond et al., 2015). The manufacturer recommends 15 to 25 animals per GEM unit for 7 days (Garnsworthy et al., 2019).

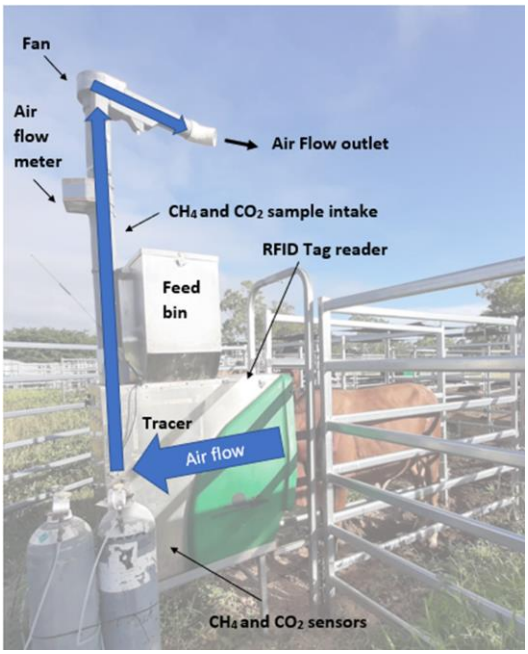


Figure 2.3. Components of the GreenFeed system for measuring methane production in ruminant animals. Adapted from Hristov et al. (2015).

2.2.2.3. Sulphur Hexafluoride Tracer Technique

The sulphur hexafluoride (SF_6) technique gives a direct measurement of the methane emissions by individual animals. This method can be utilised under normal grazing or controlled conditions where intake is measured (Goopy et al., 2016). This method uses SF_6 as a tracer gas based on the assumption that the standard SF_6 emission rate is equal to the CH_4 emission rate. A small permeation tube containing SF_6 is placed in the animal's rumen and SF_6 and CH_4 concentrations are measured near the mouth and nostrils of the animal. The gases are measured by a capillary tube placed over the nose of the animals and transferred to an evacuated collar worn around the animal's neck (Johnson et al., 1994). The enteric CH_4 emission rate can then be quantified by gas chromatography using the ratio of CH_4/SF_6 multiplied by the standard SF_6 release rate and corrected for background SF_6 concentration (Johnson et al., 1994). This technique is relatively non-invasive, cheap and valuable for studying methane emissions from a large number of individual grazing animals simultaneously (Zhao et al., 2020b). The high within and between animal variation is a significant limitation of this method and have to be taken into account to determine the number of repeated measurements to ensure accurate results (Goopy et al., 2016; Grainger et al., 2007; Pinares-Patiño et al., 2011). This method is less precise, less physically robust (high

equipment failures) and more labour intensive than respiration chambers (Goopy et al., 2016). The use of SF_6 is contradictory with the will to reduce greenhouse gas emissions as the global warming potential of SF_6 is 23900 times higher than CO_2 (Lindley and McCulloch, 2005).

2.2.2.4. Laser Methane Detector

The laser methane detector is a highly responsive, hand-held device that is pointed at an animal's nostrils measuring methane column density along the length of the laser beam (ppm.m). Generally, animals are restrained either manually or in head yokes at a feed fence for the required length of time. The operator stands at the same distance (1 to 3 m) from each animal and must keep the laser pointed at the animal's nostrils throughout the measurement period (2 to 4 min) (Garnsworthy et al., 2019). The last version of the laser methane detector showed high agreement in results with open-circuit respiration chambers (Sorg et al., 2017). This technique is non-intrusive and convenient to measure methane emissions (Zhao et al., 2020b). However, it is labour demanding (Garnsworthy et al., 2019) and expensive due to the requirement for sensitive and rapid-response instruments to analyse methane concentration and to capture micrometeorology data (Goopy et al., 2016). Moreover, under grazing conditions, further testings are necessary to quantify and exclude environmental influences such as wind speed, temperature, variabilities due to the operator (distance to the animal measured, pointing angle) and repeatability of the measurements (Sorg et al., 2017). Furthermore, DMI determination is not accurate as it is based on predictive models using the relationship between LW and LW gain (Goopy et al., 2016).

2.2.2.5. Sniffer Method

This method quantifies methane emissions from individual animals on-farm (Garnsworthy et al., 2012). Air is sampled near the animal's nostrils through a tube fixed in a feed bin and connected to a gas analyser (Garnsworthy et al., 2019). This technique has the ability to measure methane emissions for large-scale evaluation without being invasive (Garnsworthy et al., 2019). Garnsworthy et al. (2012) showed a linear relationship between the methane measured with the sniffer method and the respiration chambers (R^2=0.79). The equipment is relatively cheap depending on the gas analyser chosen

(Garnsworthy et al., 2019). Huhtanen et al. (2015) demonstrated a poor correlation between methane and CO_2 measured, DMI and LW. They also noticed a higher between-animal variation in enteric methane emissions measured using the sniffer method compared to the respiration chambers that may be due to the muzzle movement, muzzle proximity to the sample intake and variable air-mixing conditions within the feed trough. Moreover, this technique does not measure CH_4 flux or CH_4 production. The methane emissions are estimated using existing regression equations developed using the respiration chambers. Hence, different equations may be required for different dietary scenarios (Garnsworthy et al., 2012; Zhao et al., 2020b).

The correct and successful use of methane emissions measurement methods relies on the optimum matching between the objectives and the budget of the studies (Table 2.1).

Table 2.1. Methods for measuring and estimating methane emissions from cattle

Method	Indoor /grazing	Continuous/short-term	Positives	Negatives
Open circuit respiration chambers	Indoor	Continuous	- Provide most accurate and precise measurements of emissions from ruminal and hindgut fermentation - Can measure different species of animals - Measure CH_4 from hindgut and rumen	- Expensive method and labour demanding - Restricts animal behaviour and movement which may decrease feed intake - Only one animal can be measured in one chamber at one time - Animals need training
Ventilated hood / respiration head box			- Cheaper than the open circuit respirations chambers - Provide similar results to respiratory chamber	- Animals need extensive training - Only one animal can be measured by one ventilated hood at one time
GreenFeed	Indoor, grazing	Short-term	- Provide similar results to respiratory chamber and SF_6 technique. - Minimal labour input and non-invasive - Ability to measure a lot of animals	- Need enough data in a 24 h period to have an accurate representation of the diurnal CH_4 or CO_2 emissions by the animals. - Need to provide 'bait' feed - Expensive, patented device, must be purchased from C-lock Inc. and data must go through the company - Animals need training - Do not provide individual animal intake
SF_6 technique	Indoor, grazing	Continuous	- Relatively low-cost and non-invasive - Ability to measure a lot of animals - No training	- Labour demanding - Medium repeatability - High equipment failure - SF_6 is a GHG
Laser methane detector	Indoor, grazing	Short-term	- Non-invasive - Ability to measure a lot of animals - No training	- Expensive device and labour demanding - Do not provide individual animal intake - Low repeatability
Sniffer method	Indoor	Short-term	- Relatively low-cost and non-invasive - Minimum labour input - Ability to measure a lot of animals - No training	- Low repeatability - Methane concentration is not directly measured

2.3. Mitigation Techniques Against Methane Emission

2.3.1. The Use of Chemicals for Rumen Manipulation to Reduce Methane Production

2.3.1.1. The Use of Chemicals to Control Protozoa, the Main Hydrogen Producer

Some techniques such as defaunation and the utilisation of ionophores, have been used to control protozoa, the major producers of H_2 from the rumen (Joblin, 1999), so that less H_2 is accessible for CH_4 formation.

Defaunation

Defaunation techniques comprise synthetic chemicals such as copper sulphate, dioctylsodium sulfosuccinate, calcium peroxide, detergents and natural compounds, such as vitamin A, steroidal hormones or non-protein amino acids (Broucek, 2018). Dohme et al. (1999) showed that defaunation using coconut oil immediately reduced methane formation by about 40% *in vitro* using non-lactating Brown Swiss cow fed hay. However, like other inhibitors of methanogenesis, numerous defaunation agents are toxic to the animal (Mathison et al., 1998). Moreover, defaunation techniques on-farm are currently non-existent (Broucek, 2018).

Ionophores

Ionophores are classified as antibiotics and are synthetized by soil microorganisms that can modify the movement of cations, such as calcium, potassium and sodium through cell membranes. The ionophores that are particularly used to reduce methane emissions are monensin and lasalocid (Guan et al., 2006). Guan et al. (2006) showed that supplementing ionophores to 36 Angus yearling steers decreased enteric CH_4 emissions (expressed as litres per kilogram) by 30% for the first two weeks for animals on a highly concentrated diet and by 27% for the first four weeks for animals on high and low-concentrate diets, respectively. They also indicated that alternative feeding of cattle with monensin and lasalocid in comparison to only monensin did not result in further decreases or longer periods of depressed enteric methane emissions. In contrast, McCaughey et al. (1997), observed no difference in methane production in pasture-fed steers supplemented with 270 mg/d monensin controlled release capsule. According to Russell and Houlihan (2003), the possibility of transmission of antibiotic resistance from animals to

man through ionophores in animal feeds is not likely to happen. However, the use of monensin in cattle as a feed additive to increase growth and feed efficiency was phased out by the European Union Council Regulation in January 2006, but it has been re-evaluated and authorized as a feed additive for the control of coccidiosis in poultry (European Medicines Agency Veterinary Medicines and Inspections, 2007).

Another technique using probiotics has also been developed. Although the mechanism used to decrease CH_4 production is not yet clear, it may be due to the utilisation of metabolic H_2 by acetogenic bacteria to produce acetate or by decreasing the numbers of rumen ciliate protozoa (Newbold et al., 1998). Probiotics are microbial feed additives that affect fermentation in the rumen. The most widely used probiotics are yeasts such as *Saccharomyces cerevisiae* and *Lactobacillus sporogenes* (Broucek, 2018). McGinn et al. (2004) found that a commercial yeast product (procreatin-7 yeast) fed to growing beef cattle induced a 3% reduction in CH_4 production (g/g DMI). The use of probiotics appears to be an interesting method, but results have been unconvincing or yet to be confirmed *in vivo* (Martin et al., 2010).

2.3.1.2. The Use of Chemicals to Control the Methanogen Numbers

Methane inhibitors are chemical compounds with inhibitory effects on rumen archaea (Broucek, 2018). Studies using methane inhibitors such as chloroform, 3-nitrooxypropanol (3-NOP), carbon tetrachloride, methylene chloride, bromoethanesulphonate or bromochloromethane showed significant reductions in CH_4 production (Martinez-Fernandez et al., 2016; Martinez Fernandez et al., 2018; McCrabb et al., 1997; Van Nevel and Demeyer, 1996). For instance, Martinez Fernandez et al. (2018) showed that methane production (in g/kg DMI) reduced by 38% in animals supplemented with 3-NOP and by 30% for Brahman steers supplemented with chloroform compared with the control group (*Chloris gayana*). Mathison et al. (1998) indicated that methane inhibitors can reduce CH_4 emissions on short-term basis by preventing the accumulation of H_2 in the rumen, but because of microbial adaptation, the effects are rapidly neutralized and feed intake often depressed.

Overall, the utilisation of chemicals for rumen manipulation with subsequent mitigation of methane emission appears promising, but requires further development due to inconclusive results (probiotics, ionophores), microbial adaptation (defaunation, methane inhibitors) and prohibited use of antibiotics in

some countries (Broucek, 2018). The feed additive 3-NOP has not shown any side effects on the animal or the subsequent product but continues to be studied in order to be approved by regulatory bodies (Honan et al., 2021).

2.3.2. The Use of Diet Manipulation to Reduce Methane Production

2.3.2.1. The Use of Concentrates to Reduce Methane Production

Supplements are frequently used in grazing systems when availability and/or quality of pasture is limiting animal performance. To promote good animal health, supplementary feeding should satisfy the animals' needs for protein, energy, roughage and minerals. This can be a regular part of the production cycle during the dry season. The use of supplements depends on the enterprise's production objectives and seasonal conditions (Mathison et al., 1998). Table 2.2 sums up the typical tropical supplements for critical seasons used in northern Australia, often chosen for their low cost (Department of Agriculture Forestry and Fisheries, 2017).

Table 2.2. Typical tropical animal supplements for critical seasons (Department of Agriculture Forestry and Fisheries, 2017).

Animal Nutrient Needs	Supplement	Critical Season
Energy	Grains, molasses	Dry
Protein	Urea	Dry
Roughage	Silage, hay	Dry and wet
Minerals	Phosphorus	Wet

Purnomoadi et al. (2005) found that offering concentrates to Indonesian Ongole crossbred young bulls twice a day significantly reduced methane production (32.76 CH_4 g/kg DMI) compared to other bulls fed concentrate only once a day (36.33 CH_4 g/kg DMI). The same study also showed that increasing the feeding frequency of concentrates resulted in a better feed utilisation (lower feed conversion rate) and increased animal productivity with a higher daily LW gain (0.44 vs. 0.38 kg/day) Purnomoadi et al. (2005). This phenomenon can be explained by the change in fermented substrate from fibre to starch and the decline in ruminal pH, inducing a reduction in the proportion of dietary energy converted to CH_4 thereby increasing the level of concentrates in the diet (Beauchemin et al., 2008). Although increasing dietary concentrates may sometimes increase total carbon footprint by increasing the amount of emissions associated with total production, the use of pesticides, fertilisers and transportation infrastructure are indirect contributing factors (Beauchemin et al., 2008).

2.3.2.2. The Use of Legumes to Reduce Methane Production

Interest in secondary plant compounds as possible methane mitigation strategy is rising, as plant preparations are viewed as natural alternatives to chemical additives, which are prone to negative perception from consumers (Martin et al., 2010). The production of methane from rumen fermentation is generally lower with legumes than grass forages, principally due to the lower fibre content inducing a more rapid rate of passage through the rumen (Beauchemin et al., 2008).

One of the plant extracts used to reduce methane emissions belongs to the tannin families (Martin et al., 2010).

Tannins are polyphenolic compounds of plant origin. There are two main types: HT (polyesters of gallic acid and various sugars) and CT (polymers of flavonoids) as depicted in Figure 2.4 (McSweeney et al.,

2001). Tannins are broadly distributed in the plant kingdom and are known to protect against infection, insects or animal herbivory (Broucek, 2018). Tannins have the ability to form complexes with dietary proteins, minerals and polymers, such as hemicellulose, cellulose and pectin, thus delaying digestion; this confers tannins with their anti-nutritive property (Woodward et al., 2006).

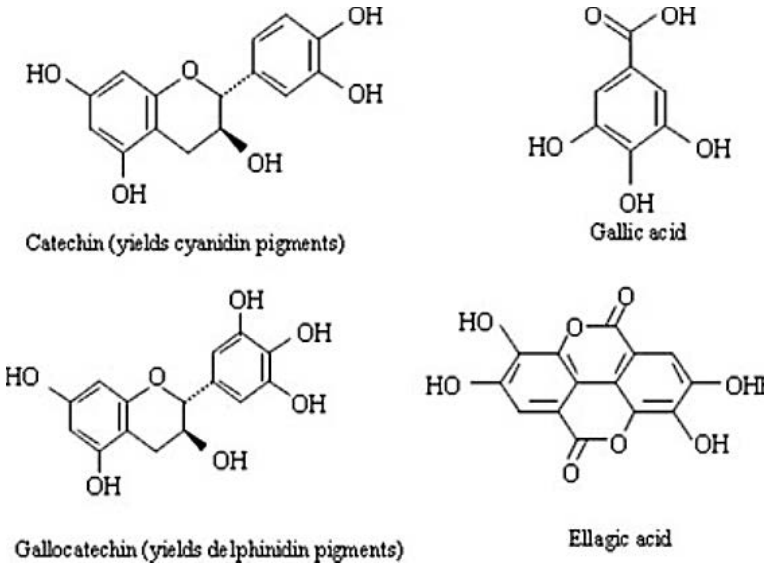


Figure 2.4. Monomeric units of condensed (catechin and gallocatechin) and hydrolysable tannins (gallic and ellagic acid) (Patra and Saxena, 2011).

Several legumes have been studied for their methane reduction properties. Hess et al. (2006) showed that extracted tannins and legumes with high tannin levels from *Calliandra calothyrsus* induced a reduction in methane emissions, but also reduced the feeding value of the diet. The same observation was made by Tiemann et al. (2008), who reported a reduction in CH_4 production by up to 24% when an herbaceous high-quality legume (*Vigna unguiculata*) was replaced with tannin-rich plants (*Calliandra calothyrsus* or *Flemingia macrophylla*). They concluded that this reduction was mainly due to a reduction in fibre digestion and organic matter.

Leucaena leucocephala, a leguminous shrub that is abundant in the tropics, contains a significant amount of CT (33 to 61 g/kg DM) (Tan et al., 2011) and a high protein content of 200 to 250 g/kg DM (Brewbaker, 1987). *Leucaena* contains mimosine ranging from 40 to 120 g/kg DM (Dalzell et al., 2012), and mimosine is an anti-nutritive compound that can be toxic at high DM intake (Dalzell et al., 2012; Tan et al., 2011). However, *in vitro* (Durmic et al., 2017; Soltan et al., 2012; Tan et al., 2011) and *in*

vivo (Harrison et al., 2015; Soltan et al., 2013) studies showed that the addition of *Leucaena* in the diet induces methane reduction. Soltan et al. (2013) conducted an *in vivo* study with Santa Inês sheep and showed that *Leuceana*, compared to Bermuda grass (*Cynodon dactylon*) in the diet, decreased CH_4 emissions and enhanced intake, body nitrogen retention, faecal nitrogen excretion and the elimination of urinary purine derivatives (a sign of the synthesis and availability of microbial proteins). In order to test the effect of tannins on methane production, they added polyethylene glycol (PEG), a tannin inhibitor, at a ratio of 1:1 PEG:*Leucaena* into the diet and did not see any significant difference in methane reduction with or without PEG. They suggested that there was no clear efficiency of tannins on methane emissions in sheep. Jones and Mangan (1977) showed that the interchange reaction of PEG with an already formed tannin-protein complex depends on the quantity of tannins and complex age before PEG addition. They explained that any increase in both factors decreases the exchange. McSweeney et al. (1999) showed that PEG addition (10 mg PEG/50 mg plant substrate) to *in vitro* fermentation can be used to analyse the effect of tannins on nitrogen digestibility. Bhatta et al. (2009) showed that tannins suppress methanogenesis by reducing methanogenic populations in the rumen by either direct inhibition of methanogens or indirect interference with the protozoal population, resulting in a decrease in the number of methanogens symbiotically associated with the protozoal population. Beauchemin et al. (2007a) found that supplementing *quebracho* tannin extract linearly decreased the proportion of acetate, resulting in a linear decrease of the acetate to propionate ratio.

The antimethanogenic activity of tannin-containing plants has been credited mostly to the condensed tannin group because hydrolysable tannins are more toxic for the animal (Beauchemin et al., 2007a). However, a study conducted by Jayanegara et al. (2015) showed that HT had a greater effect in reducing CH_4 emissions and had less negative effects on digestibility than CT. They attributed this observation to the lower risk of toxicity of CT than HT (Beauchemin et al., 2008). Ruminants consuming forage plants containing a high level of HT (*Terminalia oblongata* and the Indonesian shrub *Clidemia hirta*) showed toxicity symptoms through simple phenolics liberated in the gut (Murdiati et al., 1991). McMahon et al. (2000) reported that high tannin concentrations exceeding 40 to 50 g/kg dry matter in forages may diminish protein and dry matter digestibility in ruminants. Several experiments showed

that a level of HT lower than 20 g/kg DM did not cause detrimental effects on production parameters (Jayanegara et al., 2015). At low to moderate concentrations, CT raises dietary protein quantity, in particular, the essential amino acids. CT (polyphenolics) are able to form complexes with proteins in the rumen under the near-neutral condition of pH 6.5 and protect them from deamination, thus reducing nitrogen availability to rumen microorganisms. However, at pH 2.5 in the abomasum and abomasal end of the duodenum, the complex becomes disrupted and unstable, thereby permitting protein degradation by acidic proteases (Jones and Mangan, 1977).

In summary, legumes and plant extracts such as tannins, seem to be a good alternative for methane abatement as they are perceived to be more natural than the other methods (Martin et al., 2010). However, the addition of plant extracts does not always show conclusive results. For instance, the addition of *Leucaena* can be toxic due to high mimosine content (Dalzell et al., 2012), and Calliandra can decrease feed digestibility (Tiemann et al., 2008). Only *Desmanthus*, a tropical legume containing CT, has so far shown promising results in reducing methane emissions (Durmic et al., 2017; Vandermeulen et al., 2018) and improving animal growth performance (Aoetpah et al., 2018; Collins et al., 2016; Gardiner and Parker, 2012; Ngo, 2017; Rangel and Gardiner, 2009).

2.4. The Growth of Legumes to Increase the Pasture Quality and the Animal Performance in Northern Australia

2.4.1. The Growth of Legumes to Increase the Pasture Quality

2.4.1.1. The Legume's Ability to Fix Nitrogen

Legumes are rich in nitrogen because they have the capacity to biologically fix nitrogen and transform it into leguminous protein (Gardiner et al., 2012a). For instance, Wetselaar (1967) measured the amount of fixed nitrogen by four legumes: Townsville Lucerne (*Stylosanthes humilis*), guar (*Cyamopsis tetragonoloba*), cowpea (cv. *Poon*) and peanut (cv. *Natal common*) on Tippera clay loam for three wet seasons of growth. They showed that the total amount of N added to the soil-plant system after three wet seasons by the four legumes was 220, 220, 270 and 125 kg/ha respectively. Another study on Tippera clay loam soil in the Northern Territory displayed a higher nitrogen uptake by 30 kg/ha after

the first year, and by 55 kg/ha after the third year of maize crops on a Caribbean stylo (*Stylosanthes hamata* cv. *Verano*) legume ley compared to a grass ley (Jones et al., 1996). The presence of *Rhizobium* bacteria-legume symbioses are capable of fixing nitrogen under dry conditions, which benefits not only the legumes, but also associated grasses (Zahran, 1999).

Northern Australian graziers are concerned about the 'rundown' of buffel grass, which constitutes the dominant sown species in the area. Buffel grass pastures older than 10–20 years since establishment have declined by up to 50% in all districts. This decrease is principally related to the lack of nitrogen in the soil. Economic analysis suggests that the best solution to overcome this 'rundown' is to establish a range of adapted pasture legumes into existing grass-only pastures in order to introduce more nitrogen. Growing legumes into a grass pasture can enable a regain of 30–50% of lost production from pasture rundown and improve economic returns (Peck et al., 2011).

2.4.1.2. The Legume's Ability to Extract Moisture and Nutrients from the Soil

Legumes also have taproots that allow for moisture and nutrient extraction from deep down the soil profile. This assists with more drought tolerance, greener and productive longevity than grasses (Sturz et al., 1997). Thus, forage legumes can have significant impacts on the environment, including nitrogen fixation, improvement of soil quality, protection from water and wind erosions (Rao et al., 2015) and improvement of carbon accumulation (Peters et al., 2013).

2.4.2. The Growth of Legumes to Increase Animal Production

Studies have shown that legumes increase animal production due to improved crude protein content and feed digestibility (Gardiner, 2016; Mero and Udén, 1998). For instance, LW gains of 190 kg/head/year were observed on improved Townsville *Stylosanthes* legumes compared to 80 kg/head/year on native pastures at a stocking rate of one beast per 2.4 hectares (Winks, 1973). Bowen et al. (2016) conducted a study on 21 sites located in the Fitzroy river catchment (Queensland) across 12 commercial beef cattle properties. They showed that tropical legume forages constituted high quality diets (*Leucaena*-grass (120 and 59), lablab (115 and 59), and butterfly pea-grass (97 and 59), g CP/kg DM) and dry matter digestibility (DMD) in comparison with perennial grass pastures that had 66 g CP/kg DM and 55% DMD. These high-quality diets resulted in an annual per ha LW gain of 2.6 kg when cattle grazed

paddocks containing *Leucaena* and Butterfly peas with perennial C4 grass which was 1.6 times higher than for cattle grazing only perennial grass pastures. Coates et al. (1997) found that the introduction of legumes such as stylo pastures, improved annual LW gains (0.45 kg/day) and decreased turn-off age by at least 3–6 months and extended the growth of cattle into the late wet season and minimised dry season LW loss (Bowen et al., 2016).

Thus, it seems the addition of legumes to grass improves pasture quality and animal performance. Throughout the long annual dry seasons of northern Australia, the semiarid clay soil region has no sown pasture legumes with recognized adaptation and persistence (Gardiner and Swan, 2008). Therefore, to help meet beef cattle production requirements, farmers use nutritional supplementation strategies (CSIRO, 2007), agistment or selling stock to reduce stocking rates (MLA, n.d.).

2.4.3. The Legumes Present in Northern Australia

Northern Australian legumes such as *Crotalaria* spp., *Cullen* spp., *Glycine* spp., *Indigofera* spp., *Rhynchosia* spp., *Sesbania* spp. *and Vigna* spp. are often described as being intolerant of grazing (Clements and Henzell, 2010), toxic and/or not palatable (Gardiner, 2016). Some legumes, such as *Stylosanthes* with its cultivars Seca (*S. scabra*) and Verano (*S. hamata*) have been incorporated into native grass pastures on light textured soils, such as black spear grass (*Heteropogon contortus*). This legume has shown to be beneficial in cattle LW gains in the range of 30–60 kg/head/year and improving stocking rates (Gardiner, 2016; Hall and Walker, 2005). Though, in semi-arid northern regions with textured clay soils, also called vertisols, the stylos are not usually well adapted and few other sown legume species have shown persistence in such environments (Gardiner, 2016). *Leucaena* is another notable success in the development of exotic species in northern Australia, especially after the discovery by Raymond Jones that a bacterium (*Synergistes jonesii*) could degrade DHP (3-hydroxy-4(IH) pyridone), a breakdown product of the *Leucaena* toxic agent, mimosine (Clements and Henzell, 2010; Jones and Lowry, 1984). The search for legumes broadly adapted to the Australian subtropics had limited success. Indeed, twining tropical legumes including *C. pascuorum*, *Clitoria ternatea* (butterfly pea), Sirano (*Macroptilium atropurpureum*) and *Centrosema mole* (centro) did not persist under grazing and could not regenerate from seeds when the first-established plants died (Clements and Henzell,

2010). Some other legumes were persistent but suffered from other deficiencies, such as limited environmental adaptation to the wide range of the Australian subtropical environment, low palatability and weedy characteristics that reduced their attractiveness (Clements and Henzell, 2010). However, *Desmanthus*, a legume native to the Americas has shown persistence under heavy grazing on clay soils (Eady et al., 2011). In the 1990s, various *Desmanthus* accessions had persisted for more than two decades in abandoned trial sites across remote northern and central west Queenslands' semi-arid clay soil regions (Pengelly and Conway, 2000). The Commonwealth Scientific and Industrial Research Organisation (CSIRO) and Queensland Department of Primary Industries have introduced numerous accessions of *Desmanthus* over the past 50 years (Pengelly and Liu, 2001).

2.5. *Desmanthus* as a Potential Pasture Species for Ruminants

2.5.1. Performance Characteristics of Desmanthus

Desmanthus is included in the *Dichrostachys* group of the tribe *Mimoseae* (Luckow, 1993). It grows on a wide range of soil types from coastal sands to rocky limestone and saline soils. *Desmanthus* spp. are often selected for their persistence on heavy clay alkaline soils, but will also grow on lighter soils of neutral to alkaline pH (Cook et al., 2020). In locations such as Queensland, with an average annual rainfall of 616 mm (1900 to 2015) (State of the Environment, 2017), *Desmanthus* is well adapted, and capable of thriving in a 550–1000 mm average rainfall environment (Cook et al., 2020). It grows better in humid-tropical locations with annual average temperatures ranging from 22 to 28 °C. It can be defoliated by heavy frost, but is able to regrow from crowns when the moisture and temperature conditions are sufficient (State of the Environment, 2017). Its deep roots structure enables it to grow with stoloniferous grasses such as buffel grass (*Cenchrus ciliaris*), Bambatsi panic (*Panicum coloratum var. makarikariense*) and Queensland bluegrass (*Dichanthium sericeum*). Minor damages in seed crops by psyllid insects (*Accizia* spp.) in northern Australia and seed-eating bruchid beetles (*Acanthoscelides* spp. and *Stator* spp.) have been reported (Cook et al., 2020). Jones and Brandon (1998) studied the persistence and productivity of eight accessions of *Desmanthus virgatus* under grazing conditions at five levels of presentation yield at the end of the growing season in subtropical and subcoastal Queensland from 1989 to 1996. After surface sowing *Desmanthus* at 4 kg/ha in 1989, they found that

the yields averaged 0.7 t/ha at the highest grazing pressure and 4.7 t/ha at the lowest grazing pressure (Jones and Brandon, 1998). The best of these varieties has been selected, evaluated, propagated and commercialised by Agrimix Pty Ltd. (James Cook University's commercialisation partner, Virginia, QLD, Australia), as Progardes™ which stands for PROtein, GARdiner and *Desmanthus*; and includes new selections of the species *D. bicornutus*, *D. leptophyllus* and *D. virgatus*. The five selected cultivars are: JCU1 (*D. leptophyllus*), JCU 2, 3, 5 (*D. virgatus*) and JCU 4 (*D. bicornutus*) (Gardiner, 2016). The different species give a large collection of early to late maturity types, habits (herbaceous to suffructicose), edaphic and climatic tolerances (Gardiner et al., 2013). Progardes™ seeds have been sown in about 20,000 ha of commercial paddocks across northern New South Wales, Northern Territory and Queensland, using several sowing techniques, such as aerial seeding, seeding following a blade plough and stick raking (Gardiner, 2016). *Desmanthus* has an average crude protein content of 21% (Department of Agriculture Forestry and Fisheries, 2014) with CP contents of about 20.2% (leaf), 11.9% (stem) and 17% (pods) in the Progardes™ *Desmanthus* (Gardiner et al., 2013). In contrast, Australian native grasses (bluegrass, spear grass) have average CP contents between 10% at the beginning, and 5% at the end, of the wet season (Luckow, 1993). During the dry or winter season, *Desmanthus* dies back to the base, and each year, optimal moisture and/or temperature permitting, new stems sprout from the base (Luckow, 1993). A shallow planting depth (0.5–2.0 cm in at least 50–60 cm depth of good moist soil (State of the Environment, 2017)) and weed control have been shown to be beneficial for *Desmanthus* cultivar Progardes™ cultivation, particularly in central and southern Queensland. In general, the end of the dry season/start of the wet season is a good period to sow *Desmanthus* seeds and enable some grazing to occur late in the summer/autumn period in northern Queensland (Gardiner, 2016). However, due to unpredictable annual rainfall, it is advisable to plant 3 kg of Progardes™ seeds/ha as a combination of half-hard and half-soft (scarified) seeds. Scarification has been used in the horticultural industry to improve the rate of seed germination by chemically or physically altering the seed coat. The purpose is to increase the diffusion of water and gases into the seeds (Pandrangi et al., 2003). Scarification of Progardes™ with hot water or a mechanical abrasive disc for commercial batches enhances germination ranging from 10% to 80% (Gardiner, 2016). Its seed yield ranges between 400 and 600 kg/ha from direct harvesting (Clem, 2009; Cook et al., 2020). The

ability of *Desmanthus* to spread and become a potential weed is limited. The late flowering cultivars such as cv. Bayamo produce limited seeds and the early flowering cultivars have high seed yields resulting in high soil seed reserves. Those reserves lead to a thickening of the planted areas with a slow spread from the original plantings (Clem, 2009; Cook et al., 2020). However, hard seeds of leguminous species are known to resist digestion and can be dispersed by ruminants in faeces (endozoochory). Gardiner et al. (2012b) found that most JCU2 seeds fed to sheep passed through the animals in 48 h with only 9% of the fed seed recovered, with about 60% remaining viable.

Consequently, *Desmanthus* seems to be a promising legume in northern Australia due to its high DM productivity, seed production, tolerance of heavy grazing in alkaline, sodic, saline and heavy clay soils and its persistence in low rainfall environments (Cook et al., 2020).

2.5.2. Desmanthus as a Potential Pasture for Reducing Methane Production

As depicted in Table 2.3, Vandermeulen et al. (2018) evaluated organic matter degradability (OMD) and methane production via *in vitro* incubation of ruminal fluid from grazing Brahman (*Bos indicus*) steers on Rhodes grass (as control), *Desmanthus bicornutus*, *D. leptophyllus* and *D. virgatus* harvested from Agrimix Pty. Ltd. commercial plots. They showed that *D. leptophyllus* had a significantly lower methane emission per unit of fermented organic matter during winter in comparison to the control and other *Desmanthus* species. For instance, after 72 h of incubation, 29.56 mL CH_4/g OM (organic matter) fermented was emitted in the presence of *D. leptophyllus;* 38.72 mL CH_4/g OM was fermented for the control; and 39.90 and 32.94 mL CH_4/g OM fermented for *D. virgatus* and *D. bicornutus* respectively (Vandermeulen et al., 2018). They also found a negative correlation between HT concentration in *Desmanthus* forages and CH_4 emission per g of OM fermented. Consequently, they hypothesised that a possible anti-methanogenic property of HT (Vandermeulen et al., 2018). Nonetheless, Durmic et al. (2017) revealed mixed results regarding the potential of *Desmanthus* to reduce methane emissions *in vitro*. They compared the average methane emissions from 23 tropical grasses and showed that JCU1 could reduce methane almost all year long except in winter compared to tropical grasses. However, they showed that JCU4, JCU2 and *D. virgatus* cv. Marc increased methane emissions compared to the 23 tropical grasses all year round except in summer.

2.5.3. Desmanthus as a Potential Pasture to Increase Animal Production

Gardiner and Parker (2012) showed that steers grazing a mixed buffel grass-Progardes™ pasture in central Queensland gained an extra 40 kg LW over a 90-day period in comparison to steers on a buffel grass-only based diet during the dry season (Table 2.3). Another study conducted in central Queensland has shown that cattle grazing paddocks containing buffel grass with Progardes™ at a population density of 7 plants/m^2 had an additional gain of 40 kg/head compared to steers grazing only buffel grass (Collins et al., 2016). A 56-day feeding trial with 24 growing goats showed that supplementing animals with 40% *D. bicornutus* and sudangrass (*Sorghumbicolor*) induced an average daily gain of 60.9 g/day compared to 82.3 g/day on 40 % alfalfa (*Medicago sativa*) and 60% sudangrass (Kanani et al., 2006). However, another study on goats showed that by feeding them with JCU1 hay, the animals gained more weight (9.6 kg in 4 months) compared to the animals fed Rhodes grass with urea, Rhodes grass with urea and cottonseed meal or Rhodes grass with cottonseed meal (0.7, 5.6, 6.7 kg respectively) (Aoetpah et al., 2018). Rangel and Gardiner (2009) showed the potential advantage of providing 30% *Desmanthus* to sheep on a Mitchell grass hay diet. They observed reduced weight loss, higher feed intake and wool growth exceeding 19% over the 6-week experimental duration. Sheep showed a positive nitrogen balance and significantly enhanced weight gains and intakes by supplementing *D. leptophyllus* to a Flinders grass diet.

Table 2.3. Effects of *Desmanthus* on methane production, growth performance and rumen fermentation

***Desmanthus* Species**	**Experiment**	**Dosage**	**Control Dosage**	**Effects**	**References**
D. bicornutus, D. cv. JCU4*, leptophyllus* cv. JCU1*or D. virgatus* cv. JCU2	*In vitro* (Brahman steers rumen fluid)	1 g of organic matter of *Desmanthus* + 125 mL rumen fluid	1 g of organic matter of Rhodes grass forage + 125 mL rumen fluid	↓ ME, VFA	(Vandermeulen et al., 2018)
D. leptophyllus cv. JCU1	*In vitro* (sheep rumen fluid)	10 mL of 1:1.3 or 1:1.5 dilution of inoculum:buffer + 0.1 g freeze dried *Desmanthus*	10 mL of 1:1.3 or 1:1.5 dilution of inoculum:buffer + 0.1 g freeze dried grass	↓ ME, VFA	(Durmic et al., 2017)
D. bicornutus cv. JCU4, *D. virgatus* cv. Marc and JCU2				↑ ME, VFA	
Progardes™	Steers	Paddock with buffel grass and Progardes™	Paddock with buffel grass	↑ LW	(Gardiner and Parker, 2012)
Progardes™	Steers	Paddock Progardes™ (7 plants/m^2) and buffel grass	Paddock with buffel grass	↑ LW	(Collins et al., 2016)
D. bicornutus	Goats	40% *Desmanthus* on a dry matter basis in the diet + alfalfa	Alfalfa	↓ LW	(Kanani et al., 2006)
D. leptophyllus cv. JCU1	Goats	*Desmanthus* hay	Rhodes grass (RG) + cottonseed meal (CSM) or RG + Urea or RG + Urea + CSM	↑ LW, ↑ Intake	(Aoetpah et al., 2018)
D. virgatus, D. pubescens or *D. leptophyllus*	Sheep	30% *Desmanthus* + Mitchell grass hay based on fresh material	Mitchell grass	↑ LW, ↑ Intake, ↑ Wool growth	(Rangel and Gardiner, 2009)
D. leptophyllus	Sheep	*Ad libitum* flinders grass hay + *D. leptophyllus* or either *D. leptophyllus* or flinders grass hay		↑ LW, ↑ positive N balance with *Desmanthus*	(Ngo, 2017)

ME = methane emissions, VFA = volatile fatty acids, LW = liveweight, ↓ = decrease, ↑ = increase.

2.6. Implications, Future Research and Conclusions

Australia as the second biggest beef exporter in the world, and particularly the state of Queensland, that produced almost half of Australia's beef and veal in 2019 (MLA, 2020), is heavily reliant on the beef industry. Enteric fermentation in livestock represents three quarters of the agricultural GHG emissions

in the form of methane and nitrous oxide, and methane production represents a significant energy loss to the animal (2 to 12% of gross energy) (Australian Greenhouse Emissions Information System, 2018; Johnson and Johnson, 1995). The Australian government allocated $2.55 billion to the Emissions Reduction Fund in 2018 (Power, 2018). This was to encourage livestock producers to use innovative methods to store carbon in vegetation and soils for reducing GHG. Queensland is most concerned by enteric fermentation emissions because its beef production is the largest agricultural industry in the state (Bray and Willcocks, 2009). Its enteric fermentation coming from grazing beef cattle represents 75% of agricultural GHG emissions (Australian Greenhouse Emissions Information System, 2018) and also represents about 80% of the overall 'cradle-to-farm gate' GHG emissions (Eady et al., 2011). However, prolonged drought, high climate variability, low quality pastures and heavy textured soils in North Queensland constitute a challenge for beef cattle productivity characterised by the poor body condition of cattle (CSIRO, 2007). Selection of environmentally well-adapted and vigorous legumes that can persist in the harsh climatic conditions of northern Australia is a good solution for alleviating various nutritional problems faced by livestock in this tropical part of Australia. Legumes enable an increase in animal production due to higher protein content and digestibility in comparison to native tropical grasses (Gardiner, 2016). The roots of legumes enable ready access to deep water, introduce nitrogen in the soil and stabilize associated grasses (Zahran, 1999). The tropical legume, *Desmanthus*, seems to be a promising legume, due to its high DM productivity, seed production, tolerance of heavy grazing in alkaline, sodic, saline and heavy clay soils and its persistence in low rainfall environments (Cook et al., 2020). For future studies using *Desmanthus*, it is important to keep in mind its establishment limitations on heavy soils due to its small sized seeds that can also constitute a risk for short-term pastures (<3 years) (Cook et al., 2020). Furthermore, *Desmanthus* containing condensed tannins, showed promising results in decreasing methane emissions (Durmic et al., 2017; Vandermeulen et al., 2018) and improving animal growth performance (Aoetpah et al., 2018; Collins et al., 2016; Gardiner and Parker, 2012; Ngo, 2017; Rangel and Gardiner, 2009). The legume also seems to be a good alternative for methane abatement, because it is a better natural alternative to chemical methods and concentrate supplementation (Martin et al., 2010). However, no study has been conducted on the impact of *Desmanthus* on *in vivo* methane emissions in northern Australia. Thus, further studies should be

conducted *in vivo* to test the effects of *Desmanthus* on methane emissions from supplemented live cattle in northern Australia.

Chapter 3 : Supplementing Northern Australian Beef Cattle with *Desmanthus* Tropical Legume Reduces *In Vivo* Methane Emissions

3.1. Introduction

Agriculture accounted for 14% of Australia's greenhouse gas (GHG) emissions with enteric methane (CH_4) fermentation contributing up to 10% of its GHG in 2018 (Australian Greenhouse Emissions Information System, 2018). The state of Queensland has 11.3 million cattle which accounted for 47% of Australian beef and veal production in 2019 (MLA, 2020). GHG is the principal source of global climate change (Intergovernmental Panel On Climate Change, 2007). Therefore, mitigating the CH_4 produced by the cattle industry especially in northern Australia, would offer an opportunity to reduce the impact of GHG emissions and climate change. Approximately half of Australia's beef cattle population is found in northern Australia, which is characterised by a tropical and arid climate with rainfall occurring mainly during the wet season of November to April. The extensive grazing system in northern Australia is characterised by low animal productivity due to poor quality native pastures with low digestibility and comparatively higher CH_4 emissions than the intensive system (Charmley et al., 2008; Costa et al., 2012).

A survey focusing mainly on eight sites in semiarid clay soil regions of central-western, north, and north-western Queensland (Blackall, Barcaldine, Longreach, Julia Creek, Isisford, Yaraka, Chillagoe, and Townsville) showed that only some *Desmanthus* accessions survived and thrived among other legumes (*Stylosanthes*, *Alysicarpus*, *Centrosema*, *Chamaecrista*, *Clitoria* and *Vigna*) under harsh conditions (grazing, floods, fires, frost, droughts, and insect attacks) after two decades (Gardiner, 2016). The selection and breeding of the surviving plants from these abandoned sites have led to the development of five new cultivars of *Desmanthus* for northern Australia and similar environments: JCU1 (*Desmanthus leptophyllus*), JCU2 and JCU3 (*D. virgatus*), JCU4 (*D. bicornutus*), and JCU5 (*D. virgatus*) (Gardiner, 2016). Some of these cultivars have also shown promising results for *reducing in vitro* CH_4 emission and improving animal growth performance (Chapter 2). Vandermeulen et al. (2018) demonstrated higher anti-methanogenic potential of *D. leptophyllus* cv. JCU1 and *D. bicornutus* cv. JCU4 after 72 h of *in vitro* rumen fermentation using rumen fluid from Brahman steers compared to a

combination of Rhodes grass hay and *D. virgatus* cv. JCU2. Their study showed a 21% CH_4 reduction with JCU1, 26% with JCU4 and 5% with JCU2 when sampled in March after 51 days of regrowth, compared to Rhodes grass (Vandermeulen et al., 2018). Durmic et al. (2017) showed a potential 48% and 45% *in vitro* CH_4 mitigation in summer with JCU1 and JCU4 respectively, using sheep rumen fluid. They postulated that the observed CH_4 reduction may be caused by secondary compounds in *Desmanthus,* such as CT, HT, and total phenolics (TP) (Aboagye and Beauchemin, 2019; Naumann et al., 2013; Vandermeulen et al., 2018). Both *D. leptophyllus* and *D. bicornutus* are erect shrubs (0.4-3 m tall). *D. leptophyllus* is woody at the base and usually much branched whereas *D. bicornutus* is unbranched or occasionally 2–3 branches from the base and becomes woody at the base with age (Luckow, 1993). The CP content of *D. leptophyllus* ranges between 10.5% to 15.5% and 15% to 27% for *D. bicornutus* (Cook et al., 2020). JCU1 was selected on the basis of its persistence under grazing and plant density relative to known *Desmanthus* cultivars (Commonwealth of Australia, 2005). JCU4 is a robust early maturing plant compared to JCU1 which is late maturing (84 and 95 days for the first flowering from sowing respectively) (Commonwealth of Australia, 2005; Gardiner, 2016). Furthermore, previous studies with steers (Collins et al., 2016; Gardiner and Parker, 2012), sheep (Ngo, 2017; Rangel and Gardiner, 2009), and goats (Aoetpah et al., 2018) supplemented with *Desmanthus* showed significant LW gains. *Desmanthus* has the potential to be a promising legume for animal growth and CH_4 reduction. However, to my current knowledge, no study has been conducted to explore the *in vivo* CH_4 mitigation capability of *Desmanthus* as a supplement in tropical beef cattle on poor quality feeds. This represents a major knowledge gap that the present study intended to fill.

Therefore, the primary objective of this study was to investigate the effect of supplementing beef cattle with incremental levels of JCU1 and JCU4 (which showed a higher anti-methanogenic potential compared with JCU2 *in vitro* (Vandermeulen et al., 2018)) on *in vivo* CH_4 emissions, LW gain and rumen metabolites. The hypothesis tested was that *feeding tropical steers with incremental levels of JCU1 and JCU4 will decrease CH_4 emissions due to the presence of tannins without negatively affecting rumen metabolites and increase the animals' LW gain*.

3.2. Materials and Methods

This study was conducted at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Lansdown Research Station, Queensland, Australia (19.59°S, 146.84°E) following the Australian Code for the Care and use of Animals for Scientific Purposes (eight edition, 2013) and was approved by the CSIRO Queensland Animal Ethics Committee (permit A02/2018).

3.2.1. Animals and Treatments

Fourteen yearling Droughtmaster steers with an average LW of 296 ± 5 kg were blocked by weight and randomly allocated to three groups of 4 animals each, and one group of 2 animals (Figure 3.1). The animals were fed in individual pens. Half of the animals in each group were allocated to either *D. leptophyllus* cv. JCU1 or *D. bicornutus* cv. JCU4. Rhodes grass (*C. gayana*) hay was offered to the animals and 4 proportions of *Desmanthus* were offered to the animals as follows: 15%, 31%, 22%, and 0% of dry matter (DM). The 15% and 31% of *Desmanthus* DM periods lasted 21 days and the last two periods (22% and 0% *Desmanthus* DM) lasted 14 days due to time constraints. The adaptation period to the experimental diet was within the 10–14-day range suggested by Cochran and Galyean (1994) and considered adequate. During the 22% *Desmanthus* period, 6 animals (3 animals on each cultivar at 22% *Desmanthus*) were supplemented with polyethylene glycol (PEG, MW 4000, Chem-Supply Pty Ltd., Gillman, SA, Australia) at 160 g/kg *Desmanthus* DM to nullify the bioactivity of tannins. Although consumption problems with PEG supplement were not anticipated, the animals were nonetheless fed an increasing amount of PEG (50 g/day) for 5 days before reaching their full amount prior to the experimental period for adaptation purposes. All animals were fed ad libitum to 10% refusals over the first seven days of each period. Thereafter, intake was reduced to 90% of *ad libitum*. Methane production was measured by open-circuit gas exchange in the last 2 days of each period. Both *Desmanthus* cultivars were harvested fresh using a crop chopper (New Holland Model 38 Crop-Chopper®, Haryana, India) on alternate days from a farm located 20 min away from the research station (19.67°S, 146.96°E). The fresh *Desmanthus* was consistently harvested at 8:30 between four and six weeks of regrowth to capture the vegetative stage of maturity to minimize differences in nutritive value between the cultivars. The *Desmanthus* was stored at 5 °C in a cool room prior to feeding. Immediately

before feeding, *Desmanthus* was mixed with chopped (Roto grind model 760, Burrows Enterprises, LLC., Greeley, Colorado, USA) Rhodes grass hay. Both *Desmanthus* and Rhodes grass hay were cut to a length of 10 cm. Diets were fed once daily at 09:30–10:00 am and all experimental steers had continuous access to reticulated water and mineral block (Trace element Northern, Olsson's, Yennora, NSW, Australia).

It can be noted that at the end of Period 4, one animal was sick and could not be measured so data from only 13 were analysed.

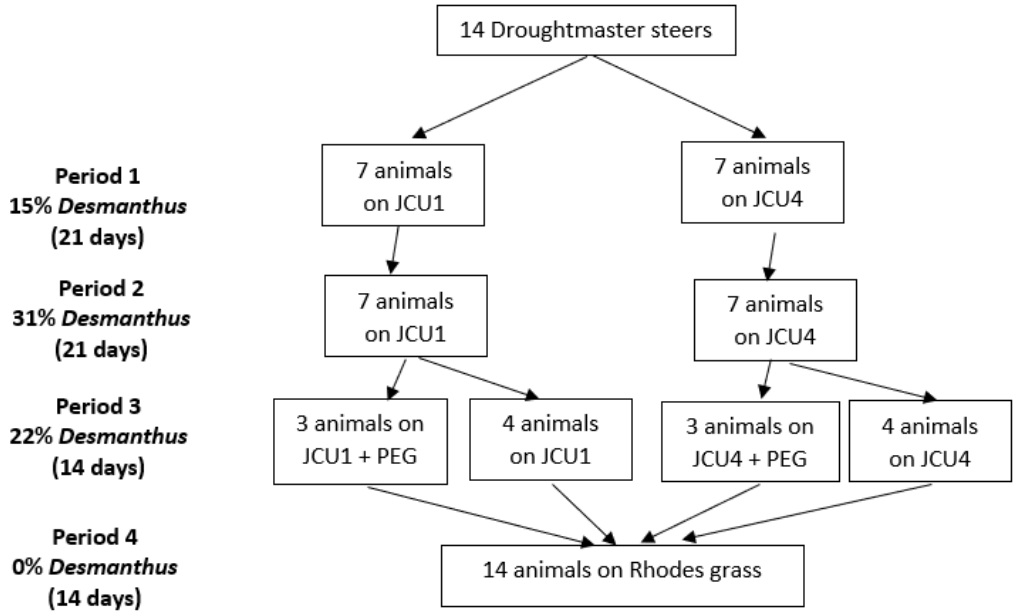


Figure 3.1. Diagram of the experimental design.

3.2.2. Feed Chemical Composition and Analysis

The DM content of the basal and experimental diets was determined by drying samples to a constant temperature at 60 °C in a fan forced oven for 48 h. The DM was calculated as the difference between the initial and final weights of samples expressed as a percentage. The oven dried samples were ground to pass through a 1-mm screen using a Tecator Cyclotec 1093 (FOSS, Hillerød, North Zealand, Denmark) for neutral (NDF) and acid detergent fibre (ADF) and total nitrogen analysis. Concentrations of NDF and ADF were measured sequentially using the filter bag method without heat stable α amylase for NDF from the operating instructions of the ANKOM 200/220 Fibre Analyzer (ANKOM Technology, Fairport, NY, USA). The analysis for total nitrogen was determined by combustion using

a Leco CN628 N Analyser (Leco, St. Joseph, MI, USA) (Sweeney and Rexroad, 1987) and the values multiplied by 6.25 to give the CP percentage. *In vitro* DMD was determined using a modified pepsin-cellulase technique described by Clarke et al. (1982). Metabolizable energy (ME) was calculated from *in vitro* true digestibility as DMD x 0.172–1.707 (CSIRO, 2007). Crude protein intake was calculated as the CP of the dry feed offered minus the CP of the dry feed refused after 24 h.

3.2.3. Extraction and Analyses of Condensed Tannins and Total Phenolics

Both *Desmanthus* cultivars were freshly sampled every week. The samples were stored at −20 °C, then freeze-dried at −50 °C for 3 days in a freeze dryer (Labogene ScanVac CoolSafe freeze dryer, Bjarkesvej 5 DK-3450, Allerød, Denmark) and ground to pass a 1-mm screen using a Tecator Cyclotec 1093 (FOSS, Hillerød, North Zealand, Denmark) and stored at room temperature (20 °C) (Terrill et al., 1992). The freeze-dried material was passed through a 0.25 mm sieve before analysis. Tannin extraction from the *Desmanthus* samples followed the procedure described by Terrill et al. (1992) except that the supernatant was diluted with distilled water to a total volume of 300 µL. Proanthocyanidin concentration (CT) was estimated by the Butanol-HCl-Fe^{III} method using purified *Desmanthus* CT as the standard with absorbance detection at 550 nm (Makkar, 2003a; Porter et al., 1985). Condensed tannins were purified on Sephadex LH-20 as described by Wolfe et al. (2008). Total phenolics concentration was determined by the Folin-Ciocalteu method with catechin as the standard (Makkar, 2003a).

3.2.4. Dry Matter Intake and Liveweight Gain

The LW of each animal was recorded weekly prior to feeding to determine the daily LW gain. The daily LW gain was calculated from the weight change over 14 or 21 days depending on the period length. Individual DMI was determined by the difference between offered and residual feed after 24 h. Individual daily intakes were recorded throughout the study to determine treatment group DMI. These values were used to calculate the DMI expressed as % of LW and to express the CH_4 yield on per kg DMI basis.

3.2.5. Rumen Collection and Volatile Fatty Acids Analysis

Rumen fluid samples were collected through an oral stomach tube using a reinforced plastic suction tube (approximately 3 cm in diameter). A hand pump was used to extract 100–200 mL of rumen fluid from the ventral sac. The rumen fluid was collected 3 h post-feeding following the second day of confinement in respiration chambers. pH of the rumen fluid was immediately measured using a pH meter and a sub-sample taken, mixed with fresh 20% metaphosphoric acid (4:1) and frozen at −80 °C for VFA and NH_3-N analyses. Rumen fluid concentrations of short chain fatty acids (acetate, propionate, n-butyrate, iso-butyrate, iso-valerate, n-valerate, and n-caproate) were measured by gas chromatography (Shimadzu Corporation, Kyoto, Japan) as described by Gagen et al. (2014). NH_3-N concentration was determined by the colorimetric method of Chaney and Marbach (1962).

3.2.6. Measurement of CH_4 Emissions

Four open-circuit respiration chambers were used to assess CH_4 production from individual steers as described by Martinez-Fernandez et al. (2016). Briefly, CH_4 emissions were measured using independent units (23.04 m^3, 3000 L/min airflow) equipped with drinking water and a feed bin containing the daily ration. The atmosphere inside the chambers was maintained at 2 °C below ambient temperature, approximately −10 Pa and a relative humidity between 50% to 75%. The exact flow rates of each chamber were corrected to measured conditions for temperature and pressure to calculate CH_4 production (Williams et al., 2007). Steers remained in the chambers for 48 h with CH_4 monitored continuously by infrared absorption (Servomex 4100, Servomex Group Ltd. Crowborough, UK). Methane production was calculated by averaging two 24 h measurements. DMI in the chamber was also recorded daily to calculate the CH_4 emissions according to feed intake (CH_4 yield expressed as g/kg DMI).

3.2.7. Statistical Analyses

All data were analysed using R (Rstudio version 1.3.1056, R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, URL http://www.R-project.org/) with the ‘dplyr’, ‘nlme’, ‘agricolae’, ‘MuMIn’, ‘car’, ’Metrics’ and ‘multcomp’ packages. Effects were considered significant at $p < 0.05$.

Multiple Analysis of variance (MANOVA) and linear mixed model procedures were conducted to compare the chemical compositions between JCU1 and JCU4 cultivars and their effects in the diet on intake, daily LW gain, CH_4 yield and products of rumen fermentation. The DM, CP, ADF, NDF, ME, TP, CT, DMI, CP intake, daily LW gain, VFA, pH, and NH_3-N were the dependent variables, while *Desmanthus* cultivars (JCU1 and JCU4) and level of *Desmanthus* in the diet were the fixed effects, and individual animals nested within blocks were the random effects.

$$Y_{ijkl} = \mu + A_i(l) + T_j + P_k + B_l + \xi_{ijkl}$$

where Y_{ijkl} is the response variable of the ith animal (i = 1 to 14) nested in the lth block (l = 1 to 4) that received the jth treatment (j = JCU1, JCU4 or 0, 15, 22, 31%) during the kth period (k = 1 to 4), μ is the overall mean of all observations, $A_i(l)$ is the random effect of the experimental animal nested in the lth block, T_j is the fixed effect of the treatment, P_k is the fixed effect of the period, B_l is the fixed effect of the block, and ξ_{ijk} is the random error component.

The same model was also used to examine the impact of supplementing with PEG on these variables except that only the data from the animals on 22% *Desmanthus* were analysed and the fixed effect was the presence or absence of PEG. The model was fitted using the restricted maximum likelihood (REML) procedure.

The same model was also used to examine the impact of DMI, percentage of *Desmanthus* in the diet, CT, TP, and CP on CH_4 production (g/day) or CH_4 yield (g/kg DMI). The model was fitted with the REML technique with the DMI, percentage of *Desmanthus* in the diet, percentage of CT, TP or CP as a fixed effect and individual animals nested within treatment groups as random effects.

When significant differences were detected, differences among means were tested by pairwise comparisons (Tukey test).

3.3. Results

3.3.1. Chemical Composition

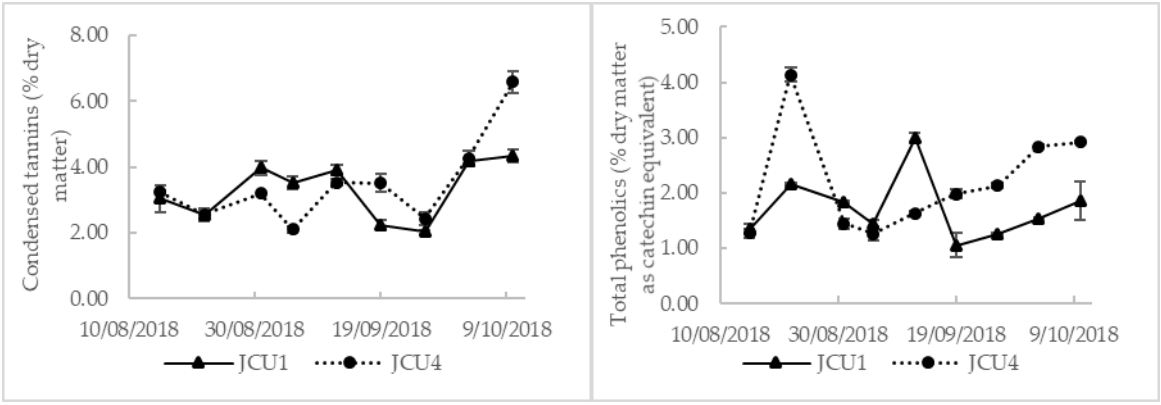
The composition of Rhodes grass hay is given in Table 3.1. Rhodes grass had a lower CP concentration than both *Desmanthus* cultivars. Rhodes grass contained less TP than *Desmanthus* and CT was not detected in the Rhodes grass. As displayed in Table 3.1, the CP, ME, and TP were higher in JCU4 than

in JCU1. JCU1 had a higher DM and fibre concentration than JCU4. There was no difference in the concentrations of CT between the two cultivars. The CT and TP in JCU1 and JCU4 were not significantly different throughout the trial (Figure 3.2).

Table 3.1. Chemical composition (means ± s.e.) of the Rhodes grass hay and the two cultivars of *Desmanthus* (JCU1 and JCU4).

Variable	Hay (*n*=28)	JCU1 (*n*=20)	JCU4 (*n*=20)
Dry matter (%)	90.9 ± 0.25	54.1 ± 1.99	42.5 ± 1.39
Crude protein (% DM)	8.2 ± 0.16	11.0 ± 0.38	14.6 ± 0.73
Acid detergent fibre (% DM)	45.0 ± 0.17	46.3 ± 0.47	36.8 ± 0.91
Neutral detergent fibre (% DM)	76.2 ± 0.27	67.4 ± 0.41	58.3 ± 0.89
Metabolizable energy (MJ/kg DM) [1]	6.4 ± 0.02	6.5 ± 0.071	7.3 ± 0.089
Total phenolics (% DM as catechin equivalent)	0.34 ± 0.03	1.7 ± 0.12	2.3 ± 0.19
Condensed tannins (% DM)	ND	3.5 ± 0.19	3.7 ± 0.30

[1] Estimated from *in vitro* true digestibility as DM digestibility x 0.172 – 1.707 (CSIRO, 2007), DM = dry matter, MJ = megajoules, ND = not detected.



(**a**) (**b**)

Figure 3.2. Variation in (a) condensed tannins (% dry matter) and (b) total phenolics (% dry matter as catechin equivalent) throughout the feeding period (*n*=18).

3.3.2. Cultivar Effects

The higher quality of JCU4 (Table 3.1), resulted in a significantly higher CP concentration in the diet (Table 3.2) and subsequently a higher CP intake of animals fed JCU4 compared to the animals fed JCU1 (0.36 ± 0.025 and 0.45 ± 0.033 kg/day for JCU1 and JCU4 respectively). The lower ADF concentration in JCU4 (Table 3.1) significantly reduced ADF concentration in the diet with JCU4 compared to JCU1 (Table 3.2). However, there was no significant difference between cultivars for DMI, daily LW gain, CH_4 yield, and products of rumen fermentation, except for the rumen concentration of iso-valerate (0.56

± 0.036 and 0.67 ± 0.034 molar % for JCU1 and JCU4 respectively) and n-valerate (0.66 ± 0.018 and 0.76 ± 0.022 molar % for JCU1 and JCU4 respectively).

Table 3.2. Nutritive value (means ± s.e.) of the treatments (JCU1 and JCU4) in the four diet levels (0, 15, 22 and 31% *Desmanthus* in the diet).

Variable	*Desmanthus* cv.	% *Desmanthus* Diet				Species *p*-Value
		0 (*n*=13)	15 (*n*=14)	22 (*n*=8)	31 (*n*=14)	
Crude protein (% DM)	JCU1	8.7 ± 0.15 [a]	8.5 ± 0.03 [a]	9.9 ± 0.52 [ac]	9.2 ± 0.52 [a]	0.0077
	JCU4	8.6 ± 0.16 [a]	8.8 ± 0.23 [a]	11.5 ± 0.67 [bc]	11.8 ± 0.35 [b]	
Acid detergent fibre (% DM)	JCU1	47.1 ± 0.50 [ab]	47.3 ± 0.61 [ab]	46.8 ± 0.71 [ab]	49.6 ± 0.59 [a]	0.037
	JCU4	46.7 ± 0.60 [b]	46.5 ± 0.71 [b]	46.1 ± 0.74 [b]	47.8 ± 0.42 [ab]	
Neutral detergent fibre (% DM)	JCU1	76.6 ± 0.57 [a]	76.9 ± 0.76 [a]	73.5 ± 0.48 [b]	74.9 ± 0.73 [ab]	NS
	JCU4	76.1 ± 0.69 [a]	77.1 ± 0.93 [a]	75.7 ± 0.51 [ab]	77.4 ± 0.58 [a]	
Metabolizable energy (MJ/kg DM) [1]	JCU1	6.2 ± 0.04 [a]	6.1 ± 0.05 [a]	6.3 ± 0.07 [a]	7.5 ± 1.01 [a]	NS
	JCU4	6.2 ± 0.05 [a]	6.1 ± 0.07 [a]	6.3 ± 0.05 [a]	8.2 ± 1.34 [a]	
Condensed tannins (% DM)	JCU1	0 [a]	0.53 ± 0.01 [b]	1.1 ± 0.01 [c]	0.92 ± 0.12 [c]	NS
	JCU4	0 [a]	0.40 ± 0.03 [b]	1.1 ± 0.16 [c]	0.87 ± 0.02 [c]	

[1] Estimated from *in vitro* true digestibility as DM digestibility x 0.172 – 1.707 (CSIRO, 2007), DM = dry matter. Means between columns and species within the same variable without the same alphabetical characters (a, b, c) represent statistical differences ($p < 0.05$). Comparisons between species (JCU1 and JCU4) for each variable are declared NS, not significant when $p > 0.05$.

3.3.3. Desmanthus Level Effects

The DMI, DMI per kg of LW, NDF intake, CH_4 production and CH_4 yield followed a linear increase pattern with an increase in the percentage of *Desmanthus* in the diet (Table 3.3). Methane yield decreased with an increase in *Desmanthus* in the diet (Figure 3.4a). Every 10% increase in *Desmanthus* intake decreased CH_4 yield by 3.3%. The addition of PEG to the 22% *Desmanthus* treatments had no influence on CH_4 yield, intake, daily LW gain and VFA except for an increase in iso-butyrate (0.61 ± 0.0282 and 0.44 ± 0.0355 molar % with and without PEG respectively) and iso-valerate (0.75 ± 0.0449 and 0.51 ± 0.0525 molar % with and without PEG respectively). PEG also significantly increased the

concentration of NH_3-N (12.8 ± 1.85 and 7.7 ± 1.29 mg/dL with and without PEG respectively). The daily LW gain was not correlated to the percentage of *Desmanthus* in the diet.

Dry matter intake was highly correlated to CH_4 production ($R^2 = 0.74$) (Figure 3.3). One kg increase in DMI per day increased CH_4 production by 47%. Methane yield followed a linear regression with the increase in the *Desmanthus* percentage in the diet (Figure 3.4). Thirty percent of *Desmanthus* in the diet decreased CH_4 yield by 10%. The increase in *Desmanthus* DMI and CT in the diet also induced a linear decrease in CH_4 yield. One kg of *Desmanthus* and 1% of CT in the diet decreased CH_4 yield by 8%. However, no correlation was found between CH_4 yield and the percentage of TP in the diet ($p = 0.22$), CP offered ($p = 0.80$) and NDF offered ($p = 0.36$).

The concentration of total VFA, acetate, acetate/propionate ratio, n-valerate, and NH_3-N significantly increased with an increase in the percentage of *Desmanthus* in the diet (Table 3.4). On the other hand, the concentration of propionate, iso-butyrate, and iso-valerate decreased with the percentage of *Desmanthus* in the diet. There was no correlation between the level of n-butyrate, n-caproate, and the rumen pH with the increasing level of *Desmanthus* in the diet**.** It can be noted that the DMI did not decrease significantly in the respiration chambers (4.3 ± 0.15 kg/day and 4.2 ± 0.09 kg/day outside and inside the respiration chambers respectively). However, the animals seemed to leave preferentially the *Desmanthus* stems out of the mixture *Desmanthus* and Rhodes grass which can impact the LW gain and methane emissions.

Table 3.3. Relationship between the dry matter intake (kg/day), DMI per kg LW (%), neutral detergent fibre intake (kg/day), daily liveweight gain (kg), CH_4 production (g/day), CH_4 yield (g/kg DMI) and the percentage of *Desmanthus* DM in the diet (means ± s.e.).

Variables	% *Desmanthus* Diet				RMSE	Linear *p*-Value	R^2
	0 (*n*=13)	15 (*n*=14)	22 (*n*=8)	31 (*n*=14)			
Dry matter intake (kg/day)	3.8 ± 0.19[a]	3.6 ± 0.17[a]	4.6 ± 0.28[b]	4.7 ± 0.26[b]	0.59	0.01	0.49
DMI/kg LW (%)	1.3 ± 0.04[a]	1.2 ± 0.05[a]	1.5 ± 0.08[b]	1.6 ± 0.06[b]	1.89	0.01	0.36
Neutral detergent fibre intake (kg/day)	3.0 ± 0.14[a]	2.6 ± 0.11[a]	3.4 ± 0.21[b]	3.6 ± 0.21[b]	0.31	0.01	0.73
Daily liveweight gain (kg)	0.018 ± 0.18	0.12 ± 0.07	0.29 ± 0.19	0.18 ± 0.07	0.42	NS	0.03
CH_4 production (g/day)	76.5 ± 2.74[ac]	68.7 ± 3.25[a]	85.6 ± 4.80[b]	81.9 ± 4.52[bc]	9.65	0.03	0.50
CH_4 yield (g/kg DMI)	19.1 ± 0.50[a]	19.2 ± 0.94[a]	18.9 ± 0.44[a]	17.5 ± 0.57[a]	2.09	0.01	0.20

Means between columns within the same variable without the same alphabetical characters (a, b, c) represent statistical differences ($p < 0.05$).

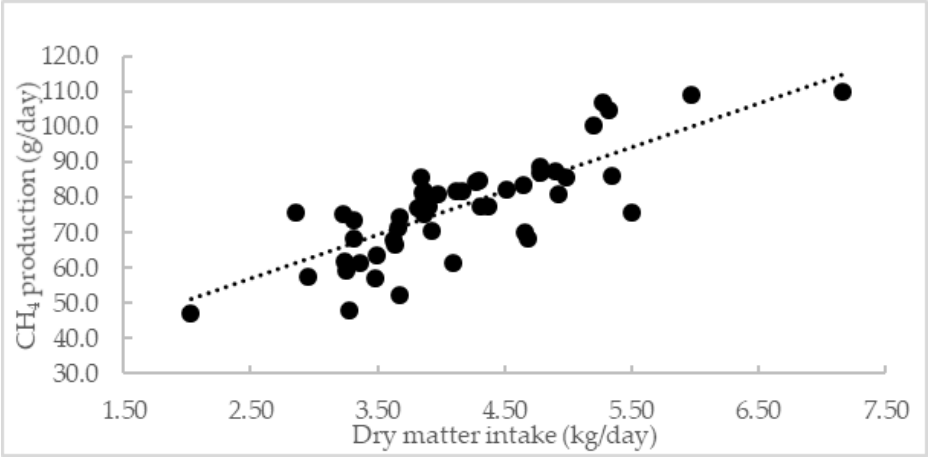


Figure 3.3. The relationship between dry matter intake (kg /day) and CH_4 production (g/day). The relationship can be described as CH_4 production (g/day) = 26.11 + 12.39x, where x = dry matter intake (kg/day) $R^2 = 0.74$, $p < 0.0001$ (n=47).

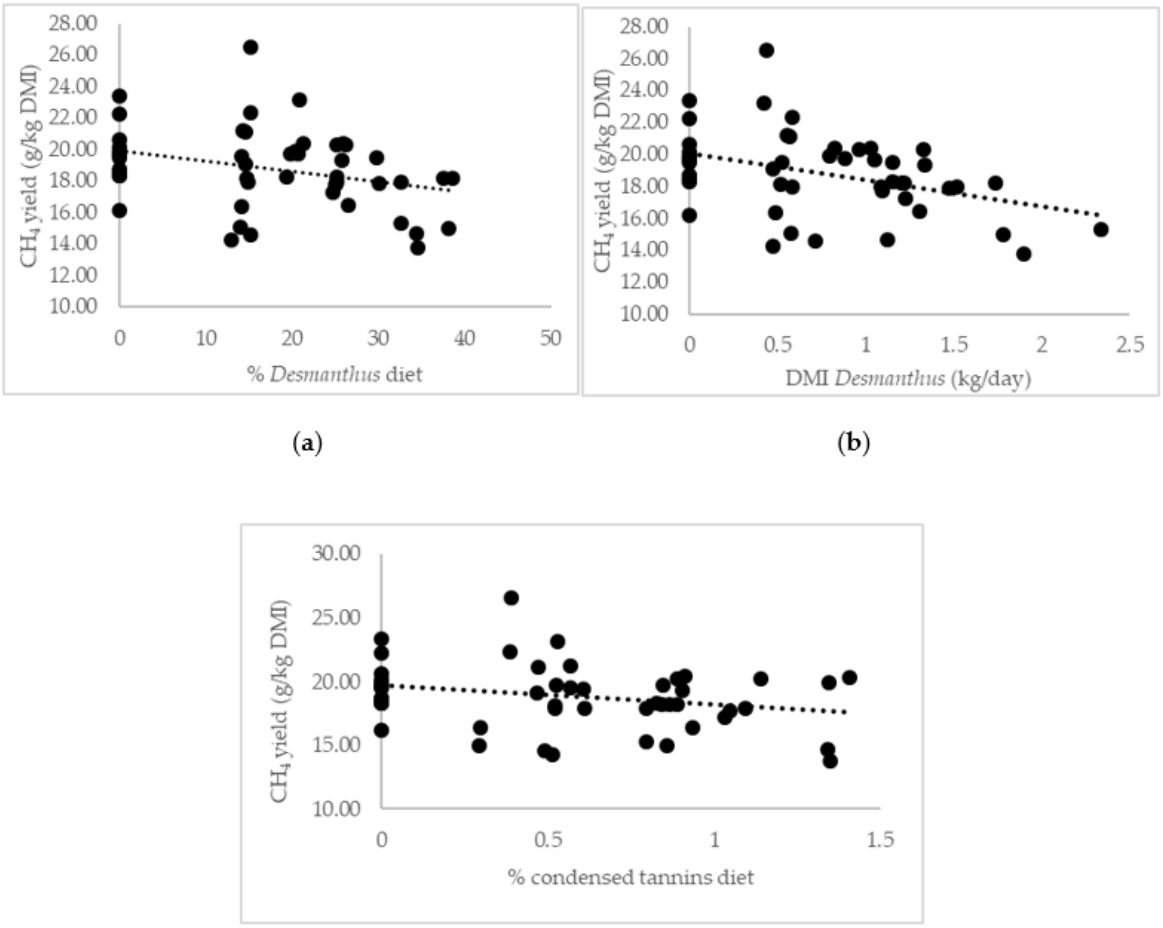


Figure 3.4. Relationship between CH_4 yield (g/kg DMI) (y) and (a) percentage of *Desmanthus* in the diet (y = 19.92 − 0.066x, where x = percentage of *Desmanthus* in the diet, R^2 = 0.20, p = 0.0097), (b) *Desmanthus* dry matter intake (kg/day) (y = 20.08 − 1.68x, where x = *Desmanthus* dry matter intake, R^2 = 0.25, *p* = 0.00075), (**c**) percentage of condensed tannins in the diet (y = 19.67 − 1.49x, where x = percentage of condensed tannins in the diet, R^2 = 0.15, *p* = 0.035) (*n*=47).

Table 3.4. Relationship between the products of rumen fermentation and the percentage of *Desmanthus* (on dry matter basis) in the diet.

Variables	% *Desmanthus* Diet				RMSE	Linear *p*-Value	R^2
	0 (*n*=13)	15 (*n*=14)	22 (*n*=8)	31 (*n*=14)			
Total VFA (**mM**)	49.8 ± 1.38	68.8 ± 3.44	55.0 ± 2.77	74.3 ± 4.41	11.25	0.01	0.36
Acetate (molar %)	70.6 ± 0.34	74.4 ± 0.45	71.9 ± 0.28	73.9 ± 0.14	1.74	0.01	0.19
Propionate (molar %)	19.4 ± 0.22	16.4 ± 0.32	18.0 ± 0.19	16.5 ± 0.11	1.19	0.01	0.30
Acetate/Propionate ratio	3.7 ± 0.06	4.6 ± 0.12	4.0 ± 0.06	4.5 ± 0.04	0.40	0.01	0.22
Iso-butyrate (molar %)	0.52 ± 0.02	0.50 ± 0.03	0.44 ± 0.03	0.43 ± 0.02	0.090	0.01	0.21
n-butyrate (molar %)	8.2 ± 0.19	7.3 ± 0.22	8.3 ± 0.14	7.7 ± 0.09	0.70	NS	0.03
Iso-valerate (molar %)	0.66 ± 0.05	0.64 ± 0.05	0.51 ± 0.05	0.58 ± 0.04	0.13	0.04	0.32
n-valerate (molar %)	0.52 ± 0.02	0.68 ± 0.02	0.71 ± 0.04	0.74 ± 0.03	0.082	0.01	0.51
n-caproate (molar %)	0.13 ± 0.01	0.11 ± 0.01	0.18 ± 0.01	0.13 ± 0.01	0.036	NS	0.13
NH_3-N (mg/dL)	6.4 ± 0.55	6.6 ± 0.41	7.7 ± 1.29	8.0 ± 0.49	2.77	0.03	0.16
pH	7.0 ± 0.06	6.9 ± 0.06	7.1 ± 0.07	6.9 ± 0.05	0.20	NS	0.15

VFA = volatile fatty acids. Values are means ± s.e., RMSE = root mean square error. Means between the *Desmanthus* level in the diet for each variable are declared NS, not significant when $p > 0.05$.

3.4. Discussion

3.4.1. Chemical Composition

D. bicornutus (JCU4) is an early maturing suffruticosa plant (Gardiner, 2016) compared to *D. leptophyllus* (JCU1) (Luckow, 1993). The quality variation between JCU1 and JCU4 might also be due to species differences where the CP in the current trial averaged 11.0% and 14.6% for JCU1 and JCU4, respectively. Cook et al. (2020) found that the CP of *D. leptophyllus* ranged from 10.5% to 15.5% compared to 15% to 27% for *D. bicornutus*. Despite their chemical compositional differences, JCU1 and JCU4 had a similar CT concentration (3.5% and 3.7%, respectively), although the TP concentration of JCU4 (2.3% as catechin equivalent) was significantly higher than that of JCU1 (1.7% as catechin equivalent). Vandermeulen et al. (2018) reported similar CT concentrations of 3.7% and 4.0% (expressed as leucocyanidin equivalent) for JCU1 and JCU4, respectively. They also reported a higher TP concentration in JCU1 (8.7% as tannic acid equivalent) than in JCU4 (7.3% as tannic acid

equivalent). Gonzalez-V et al. (2005) demonstrated that there was a lower concentration of tannins in stems than leaves. This could explain the lower TP concentration in JCU1 which was more mature than JCU4 with fewer leaves. Naumann et al. (2013) reported a CT concentration of 8.1% in *D. illinoensis* using a species-specific standard compared to the finding of Gonzalez-V et al. (2005) who reported a tannin concentration of 1.7% in *D. illinoensis* using a vanillin-HCl method and catechin equivalent (Burns, 1971). The latter found a tannin concentration of 2.1% as catechin equivalent for *D. virgatus* which was lower than the 8.9% catechin equivalent reported by Ramirez et al. (2000) for the same *Desmanthus* species using the Burns (1971) method modified by Price et al. (1978). These differences in tannin concentration show that even if species-specific CT as the internal standard is used as the most appropriate option for CT analysis (Martin, 2016; Schofield et al., 2001), the tannin results can only be useful in determining relative differences between *Desmanthus* cultivars throughout the experiment rather than for producing absolute quantitative values. Furthermore, the results can vary between laboratories depending on the standards used, presence or absence of water, impurities, light, temperature and time of color development (Wolfe et al., 2008).

3.4.2. Cultivar Effects

The results showed a higher CP intake when the animals were fed JCU4 compared to JCU1, probably due to the higher CP concentration in JCU4 (Table 3.1). However, no cultivar effect was observed on DMI, daily LW gain, pH, rumen metabolites, and NH_3-N rumen concentrations. The similar CH_4 yield results detected between the two cultivars were in agreement with the *in vitro* study conducted by Vandermeulen et al. (2018) where they found that expressed per g of fermented OM, CH_4 production in JCU1 and JCU4 were not significantly different. Looking at the overall data of the three sampling periods (March, August, and October) studied, Vandermeulen et al. (2018) also showed similar *in vitro* acetate/propionate ratios to my study (4.4) for JCU1 and JCU4 (5.0 and 4.8 respectively). In my study, the concentrations of n-valerate and iso-valerate were significantly higher in the rumen of the animals fed JCU4 than JCU1. As reported by Hristov et al. (2004), the concentration of valerate and branched-chain VFA were increased or tended to be increased with the increase of dietary N as branched-chain VFA are derived from branched-chain amino acids (Wolin et al., 1997). Consequently, the

concentration of iso-valerate and n-valerate were higher in the animals fed JCU4 compared to the ones fed JCU1 due to the higher CP intake for the animals fed JCU4.

Therefore, it seems that the differences in chemical composition between the two *Desmanthus* species had no major effects on animal performance, CH_4 emissions, and rumen function.

3.4.3. Animal Performance

Compared with other studies that showed an increased LW gain with *Desmanthus* in cattle (Collins et al., 2016; Gardiner and Parker, 2012), sheep (Ngo, 2017; Rangel and Gardiner, 2009), and goats (Aoetpah et al., 2018), my results showed a low intake and animal performance due to the poor-quality diet. Restricted CP availability has been described as the critical threshold for suitable microbial growth on the fibrous carbohydrates in basal forage which induces a decrease in animal performance and intake (Hennessy et al., 1983; Leng, 1990). Detmann et al. (2014) estimated the concentration of CP in the diet to the apparent equilibrium point where the efficiency of nitrogen utilisation in the animal's body is nil to be 10.8% DM. Yet, in my study, that level was only reached at 22% and 31% of JCU4 in the diet. Moreover, according to the EDGE manual (McLennan, 2015), cattle at 300 kg LW will be at maintenance for a diet with an ME of 7 MJ/kg DMI, and a DMI/kg LW of 1.8% which means that the maintenance requirement will be 37.8 MJ/day. Thus, in my study, with a ME of the diet averaging 6.6 MJ/kg DMI and a DMI/kg LW of 1.4%, meaning an ME intake of 27.7 MJ/day, the low animal performance was expected with a daily LW gain of 0.2 kg/head as the ME sufficiency ratio was 73%. Furthermore, the high fibre content of the Rhodes grass hay used (NDF = 76.2% DM) could have depressed the rumen microbial digestion of roughages as Dixon (1999) showed that roughages of low N content, high fibre content and low digestibility are likely to be most affected by a depression in rumen microbial digestion.

The low-quality diet also had an impact on the rumen NH_3-N concentration as the CP concentration in the diet is correlated to the NH_3-N concentration (Brandao and Faciola, 2019; Detmann et al., 2009). Ensuring adequate rumen NH_3-N concentration to supply the majority of N for supporting microbial growth is the first priority in optimizing fermentative digestion of forage (Leng, 1990). Satter and Slyter (1974) suggested the optimal ruminal NH_3-N concentration for maximal microbial growth to be 5

mg/dL. However, a more recent study conducted by Detmann et al. (2014) showed that a rumen NH_3-N concentration of 6.3 mg/dL was needed to reach the equilibrium efficiency of nitrogen utilisation. Although the NH_3-N concentration 3 h after feeding increased with an increase in *Desmanthus* in the diet, the concentration was just above the optimal concentration at 0% *Desmanthus* in the diet (6.4 mg/dL).

Previous studies considered the presence of tannins in feed as anti-nutritive, due to its negative effects on intake, digestion and absorption of nutrients and subsequently animal performance (Kumar and Singh, 1984). Vandermeulen et al. (2018) showed a decrease in organic matter digestibility (OMD) *in vitro* with JCU1 compared to JCU4, possibly due to the lower concentration of HT in JCU4 compared to JCU1. The anti-nutritive properties and toxicity of tannins are frequently attributed to a high HT ingestion due to its poorer protein absorption and release of metabolites in the rumen causing cellular damage (Murdiati et al., 1991). Unpalatability due to astringent tannins can lead to reduction in voluntary feed intake (Kumar and Singh, 1984). The optimal tannin concentration level in which intake is reduced has not been definitively determined. For instance, Grainger et al. (2009) showed a decrease in intake when cows were fed a CT concentration of 0.86% and 1.5% of DM, while Dschaak et al. (2011) showed a decrease in intake with a CT extract concentration of 3% of DM. In the present study, the addition of PEG showed an increase in NH_3-N and iso-butyrate and iso-valerate concentrations in the rumen in agreement with previous *in vitro* studies with rumen cattle fluid (Bhatta et al., 2013; Bhatta et al., 2009; Pellikaan et al., 2011). The increase in NH_3-N concentration is attributed to the inhibition of microbial deaminase by tannins, thereby inducing high protein degradability. The lower concentration of iso-acids in the presence of tannins was attributed to the ability of tannins to bind proteins and the subsequent protection from ruminal deamination as iso-acids are derived from amino acids catabolism in the rumen (Bhatta et al., 2009; Fagundes et al., 2020a; Hristov et al., 2004). Therefore, a reduction in protein degradation in the rumen will increase the quantity of protein digested in the small intestine (Patra and Saxena, 2011). Even if the presence of tannins in *Desmanthus* protected the proteins from degradation in the rumen, it did not seem to have an impact on total VFA, daily LW gain and DMI. The linear increase in DMI with increases in *Desmanthus* and CT from 0% to 1.1% in

the diet suggests that the tannin levels in the current study were not toxic to ruminal microbes and did not have a negative impact on animal performance.

It seems that the low-quality basal diet with low CP and high fibre was the major reason for the low animal performance observed in the present study. The low-quality feed in the present study (8.2% CP in the Rhodes grass hay) was chosen to mimic the low-quality feed base in northern Australia. Poppi et al. (2018) reported a dietary CP concentration below 6% for about nine months of the year in Mitchell grass (*Astrebla spp.*), a native Australian species. They also reported a CP below 6% for about two months and a CP averaging 8% six months of the year in the introduced buffel grass (*Cenchrus ciliaris*) pastures.

3.4.4. Effect of Desmanthus Level on CH_4 Emissions

The non-significant difference in CH_4 emissions between the two *Desmanthus* cultivars corroborates the *in vitro* report of Vandermeulen et al. (2018) where they found a similar CH_4 production between JCU1 and JCU4 in March and October. In contrast, these authors reported a higher CH_4 emission in August with JCU4 compared to JCU1, as did Durmic et al. (2017) using *in vitro* techniques.

Methane production was highly correlated to DMI ($R^2 = 0.74$) (Figure 3.2) as shown in other studies (Charmley et al., 2016), therefore CH_4 yield was chosen to better understand the mechanism behind CH_4 reduction. Methane yield followed a linear increase pattern with an increase in *Desmanthus* percentage in the diet (Figure 3.4a). The coefficient of determination was higher when the CH_4 yield was expressed as a function of *Desmanthus* DMI (Figure 3.4b). The observation that the addition of *Desmanthus* in the diet reduces CH_4 emissions agrees with *in vitro* data by Vandermeulen et al. (2018). They showed a CH_4 abatement of 21% and 26% after 72 h *in vitro* incubation with Brahman cattle rumen fluid for JCU1 and JCU4, respectively compared to Rhodes grass. Durmic et al. (2017) revealed mixed results regarding the potential of *Desmanthus* to reduce CH_4 emissions. In comparison to the average CH_4 emissions from 23 tropical grasses, they showed that JCU1 generally reduced CH_4, whereas JCU4 generally increased CH_4 emissions.

Vandermeulen et al. (2018) reported a negative correlation between CH_4 production (mL/g OM) and TP, total tannins, and CT in *Desmanthus* and a highly significant correlation between HT and CH_4

production *in vitro*. Previous studies also showed a reduction in CH_4 in the presence of tannins *in vitro* and *in vivo* (Aboagye and Beauchemin, 2019; Jayanegara et al., 2012). In the present study, CH_4 yield as a function of the percentage of CT in the diet, followed a linear regression pattern (Figure 3.4c). However, no correlation was found between CH_4 yield and percentage of TP in the diet ($p = 0.22$). These results are in discordance with the study conducted *in vitro* by Jayanegara et al. (2010) using 17 polyphenol-containing plants where they reported a significantly negative relationship between CH_4 production and TP and tannins, but not with CT. They concluded that TP and total tannins were good predictors of CH_4 reduction potential. Tannins affect rumen microbial ecology and metabolism (McSweeney et al., 2005), but the mechanisms by which they affect methanogenesis are yet to be defined (Aboagye and Beauchemin, 2019). Some studies suggested that the greater the tannin molecular weight, the greater the CH_4 reduction as the ability to bind methanogens would be higher (Petlum et al., 2019; Saminathan et al., 2016). Condensed tannins have a high molecular weight (1900 to 28,000 Da) compared to HT (500 to 3000 Da) (Aboagye and Beauchemin, 2019). Naumann et al. (2013) did not find any correlation between the molecular weight of CT and CH_4 emissions. Furthermore, Naumann et al. (2014) reported no correlation between protein-precipitable phenolics or the amount of bound protein and the molecular weight of CT, although they showed a correlation between the CT, protein-precipitable phenolics and bound protein. Aboagye et al. (2019) showed that gallic acids in HT had the potential to lower CH_4 and nitrous oxide emissions in beef cattle without reducing feed digestibility due to their ability to bind and precipitate proteins (Zeller, 2019).

To my knowledge, only one study (Naumann et al., 2014) analysed *D. illinoensis* and reported the molecular weight of CT (866 Da). Jayanegara et al. (2015) suggested that the measure of biological activity of tannins is more accurate than measuring the concentration of tannins in the plant when it comes to studying the CH_4 mitigation potential of tannins. Vandermeulen et al. (2018) added that the verification must use Rubisco as the model protein because it represents the principal protein in fresh fodder at rumen pH 7 (McAllister et al., 2005).

It should be noted that in the present study, the increase in tannin concentration was due to the increase in *Desmanthus* concentration in the diet and not due to an increase in tannin content in the cultivars. Vandermeulen et al. (2018) reported similar or lower HT than CT in their study. Herein, the amount of

PEG added in the diet (3.2 g PEG/g CT for the highest level of CT in the diet) was higher than the optimum concentration of PEG (1.2 g PEG/g tannin) suggested by Makkar et al. (1995) to have a response in CH_4 emission. However, in the present study, the addition of PEG had no impact on CH_4 yield. This finding disagrees with previous *in vitro* and *in vivo* studies (Animut et al., 2008; Bhatta et al., 2013; Bhatta et al., 2009). Bhatta et al. (2009) found a 6.5% decrease in CH_4 output *in vitro* when cattle rumen fluid was incubated with 25% tannin on a DM basis without PEG. Animut et al. (2008) also showed an increase in CH_4 production *in vivo* in goats ranging from 9.6 L/day to 19.0 L/day when a diet containing tannins at 15% of DM was not supplemented with PEG. However, another *in vitro* study (Bhatta et al., 2013) comparing 21 medicinal and aromatic plant leaves as antimethanogenic additives in bull feeds, showed mixed results. They found the highest CH_4 increase with PEG addition (57%) with *Clerodendrum inerme* containing a low tannin concentration (0.03% DM CT as leucocyanidin equivalent and 2.4% DM HT) whereas the *Terminalia cordifolia* containing the highest concentration of tannins (1% DM CT as leucocyanidin equivalent and 25.2% DM HT) showed an increase in CH_4 with PEG addition of only 7.2%. With respect to the tannin effect depending on the plants, it seems that the effect of *Desmanthus* tannins in CH_4 may have been less than with other plants. It is possible that the anti-methanogenic effect observed in my trial was due to the response of the rumen to improve nitrogen availability.

The increase in NDF intake with the increasing level of *Desmanthus* might be due to the increase in DMI and the relatively constant NDF concentration with the increasing level of *Desmanthus.* The lack of correlation between the CP or NDF offered in the diet and CH_4 yield (g/kg DMI) in the present study is in contradiction with previous studies that reported significant correlations between diet quality and CH_4 emissions (Ramin and Huhtanen, 2013; Singh et al., 2016). This observation would support the role of tannins in reducing CH_4 emissions rather than the higher diet quality induced by an increase of *Desmanthus* in the diet.

3.4.5. Effect of Desmanthus Level on Rumen Metabolites

The increase in total VFA concentration with increasing levels of *Desmanthus* and thus tannins in the diet, was contrary to the effect observed in some studies in which a reduction in VFA was observed

with the addition of tannins to the diet (Jayanegara et al., 2015; Vandermeulen et al., 2018). Similar results as in the present study were observed by Avila et al. (2020) and Dickhoefer et al. (2016). They associated the increase in VFA to the reduction in water intake in treatments containing CT rather than an effect on carbohydrate degradation. The increase in rumen VFA may also be due to the increased supply of fermentable organic matter in the form of protein-N because the amino acids resulting from proteolysis can be deaminated and the carbon skeletons formed as a result can be fermented to VFA (Brandao and Faciola, 2019; Vanegas et al., 2017).

Methane and propionate are usually negatively correlated due to competition for hydrogen (Bhatta et al., 2009). The formation of acetic and butyric acids induces the production of H_2 and CO_2, whereas propionic acid production requires a net uptake of H_2 resulting in a decrease in methanogenesis (Benchaar et al., 2001). The present study showed the opposite trend with an increase in acetate and a decrease in propionate as the level of *Desmanthus* in the diet increased. This might be due to the high concentration of NDF which stayed relatively constant, even with an increase in *Desmanthus* in the diet (76% DM). As dietary NDF increases, so does the molar proportion of acetate and subsequent decrease in the proportion of propionate (Brandao and Faciola, 2019).

3.5. Conclusions

Desmanthus leptophyllus (JCU1) and *Desmanthus bicornutus* (JCU4) showed that irrespective of cultivar, incremental supplementation with *Desmanthus* level in the diet induced a linear decrease in CH_4 production and increase in VFA concentration. The ability of tannins in *Desmanthus* to reduce CH_4 emissions with the addition of PEG in the diet was absent albeit CH_4 yield was negatively correlated with CT in the diet. Nevertheless, supplementation with PEG increased rumen NH_3-N and iso-acid concentrations, suggesting an effective ability of tannins in *Desmanthus* to bind rumen proteins. It was also apparent in this study that increasing the *Desmanthus* level in the diet, increased the DMI without increasing the daily LW gain. The hypothesis that, on a low-quality diet, feeding tropical steers with incremental levels of *Desmanthus* will decrease CH_4 emissions due to the presence of tannins without negatively affecting rumen metabolites and increase the animals' LW gain is partly true. Therefore, it is concluded that *Desmanthus* has a potential to maintain the LW of the animals and reduce *in vivo* CH_4 emissions by beef cattle in the drier parts of northern Australia possibly due to a combination of rumen

fermentation and tannin effects. These findings could contribute to the larger global effort of reducing the impact of climate change and greenhouse gas emission. However, further *in vivo* investigation is needed to better understand the mechanism behind the observed CH_4 reduction associated with *Desmanthus* supplementation in the diet.

3.6. Summary

The main objective of this study was to investigate the effect of supplementing beef cattle with incremental levels of *Desmanthus leptophyllus* cv. JCU1 and *Desmanthus bicornutus* cv. JCU4 on *in vivo* CH_4 emissions and the role of tannins in rumen fermentation. Fourteen yearling Droughtmaster steers were allocated to each of the two *Desmanthus* species and offered a basal diet of Rhodes grass (*Chloris gayana*) hay plus fresh *Desmanthus* at 0%, 15%, 22%, and 31% of DMI. The 15% and 31% *Desmanthus* periods lasted 21 days and the 22 and 0% *Desmanthus* periods, 14 days. Methane production was measured by open-circuit gas exchange in the last two days of each period. The results showed a linear increase in DMI and reduction in CH_4 yield with the increasing level of *Desmanthus* and subsequently condensed tannins in the diet. The added tannin binder polyethylene glycol-4000 did not affect CH_4 yield but increased rumen NH_3-N and iso-acid concentrations. Therefore, on a low-quality diet, *Desmanthus* has the potential to increase intake and reduce CH_4 emissions. Even though its tannins can bind rumen proteins, the beef cattle anti-methanogenic response to supplementation with *Desmanthus* may be a combination of rumen fermentation and tannin effects.

Chapter 4 : Plasma Metabolites, Productive Performance and Rumen Volatile Fatty Acid Profiles of Northern Australian *Bos Indicus* Steers Supplemented with *Desmanthus* and Lucerne

4.1. Introduction

"Metabolomics" as a research discipline, is a term derived from "the study of metabolites", which comprehensively measures the end-products (small molecule metabolites) of complex *in vivo* metabolic processes such as glucose, urea, NEFA, bilirubin, aspartate aminotransferase (AST), etc., in cells, biofluids and tissues using advanced analytical chemistry techniques. The blood metabolome provides a suite of predictive biomarkers for livestock health, productive performance, and disease monitoring because cattle go through physiological and metabolic adjustments during growth and development (Connolly et al., 2020; Goldansaz et al., 2017). Recent research investigations in metabolomics have generated compelling results showing that metabolites such as glucose and urea can help farmers and the livestock industry to evaluate dietary responses to different feeds, and this makes metabolomics an ideal tool for livestock research (Goldansaz et al., 2017). For instance, in fattening Holstein bulls, Yang et al. (2021) reported that plasma ammonia (NH_3) was a metabolic waste product and any increase in its circulation due to inefficient nitrogen (N) conversion to amino acids would affect animal health and growth performance. In Wagyu crossbred steers, Connolly et al. (2019) found that blood metabolites were either positively or negatively correlated with key production traits including growth rate, carcass weight, and subcutaneous and intramuscular fat, thus potentially offering biomarkers that could be used for individual steer selection for feedlot performance. Whereas published reports on the impacts of seasonal and dietary nutrient supplementation on rumen microbiota structure and metabolites of beef cattle abound (Kim et al., 2020; Martinez-Fernandez et al., 2020; Zhao et al., 2020a), to our current knowledge of the published literature, there are no existing peer reviewed reports on the plasma metabolite profiles of tropical northern Australian beef cattle steers supplemented with the tropical legume *Desmanthus.* Our present research was intended to fill this knowledge gap.

The Northern Australian beef industry is defined by an extensive grazing system in dry tropical rangelands. In this particularly low N environment, undernutrition is the major issue, especially during the dry winter season (Martinez-Fernandez et al., 2020). Animals that efficiently convert feed into meat or body mass have a high difference between their actual feed intake and the expected feed requirements for maintenance and growth (low residual feed intake, RFI) over a particular time period (Foroutan et al., 2020a). A recent study demonstrated that feeding cattle with an N-supplemented diet in this environment enhanced rumen fermentation and increased bacterial populations involved in pectin and hemicellulose degradation and ammonia assimilation (Martinez-Fernandez et al., 2020). This change in the rumen microbiota structure induced a lower RFI with an increase in the daily LW gain, rumen NH_3-N, butyrate, and the acetic to propionic acid ratio (Martinez-Fernandez et al., 2020). High quality feeds have been shown to increase plasma urea which serves as a promising and inexpensive metabolite to predict and categorize bovine RFI values (Fitzsimons et al., 2013; Foroutan et al., 2020a). Additionally, dietary proteins in roughages and N utilisation are fundamental to growth and development in ruminants, but an estimated 70% of ingested N is excreted as urinary and faecal N which can limit animal productive performance and cause environmental pollution (Yang et al., 2021). A decrease in faecal and urinary N implies a higher N retention and a more efficient regulation of the urea cycle and conversion of rumen NH_3-N. Therefore, a better understanding of energy and protein metabolism through an assessment and synthesis of rumen VFA, branched-chain fatty acids (iso-acids) and plasma metabolites in steers supplemented with *Desmanthus* relative to lucerne (*Medicago sativa*), will provide baseline information on NH_3-N synthesis, urinary and faecal N excretion, and hence N retention and utilization for growth and LW gain.

Lucerne is considered as one of the main perennial legumes in the world with an estimated world cropping area of 30 million ha (mainly located in North America, Europe, and South America), and an extensive use for ruminant livestock feeding systems in temperate Australia (Annicchiarico et al., 2015). It is globally used due to its high quality CP content ranging between 14 and 24% on a DM basis (Kanani et al., 2006; Le et al., 2019; McDonald et al., 2003; McDonnell et al., 2017), which induces an increase in animal production. McDonald et al. (2003) stated that in the Australian southern state of New South

Wales, sheep wool and cattle LW increased by 10–20% when lucerne was included in pastures based on subterranean clover or phalaris. Lucerne is not adapted to most central Queensland soils (Pengelly and Conway, 2000) because of its intolerance to saline soils (Annicchiarico et al., 2015) and failure to persist in sufficient plant densities for more than 2 years (Pengelly and Conway, 2000). In contrast, Chapter 2 on the use of *Desmanthus* (JCU1 cv. *D. leptophyllus* and JCU4 cv. *D. bicornutus*) for beef cattle production in Northern Australia showed that *Desmanthus* as a tropical legume, not only persists and survives under harsh tropical conditions, but also contains high CP levels that elicited promising LW gains in steers, sheep, and goats. Kanani et al. (2006) reported that supplementing goats with 40% *Desmanthus bicornutus* and 60% Sudangrass (*Sorghum bicolor*) induced a daily LW gain of 60.9 g compared to 82.3 g on 40% lucerne and 60% Sudangrass. However, *Desmanthus* contains tannins which are polyphenolic molecules that have the ability to form complexes with proteins, and to a lesser extent, with metal ions, amino acids, and polysaccharides (Makkar, 2003b). Tannins have been shown to improve N utilisation by decreasing rumen degradability of CP and sometimes CP digestibility in the digestive tract which shifts N loss from urine to faeces (Grainger et al., 2009; Lagrange et al., 2020; Tseu et al., 2020). Faecal N is mainly in the organic NH_2 form which has to be mineralized to ammonium (NH_4^+) before it can volatilize or leach. The CT-protein complex inhibits this mineralization process by slowing down the breakdown of protein from faeces to NH_4^+ (Lagrange et al., 2020). More than 70% of urinary N is in urea form which is readily available for hydrolysis and conversion into NH_4^+ (Sordi et al., 2014). The nitrification of NH_4^+ to nitrate (NO_3^-) produces the gaseous forms of nitric oxide (NO), nitrous oxide (N_2O), or dinitrogen (N_2) with the reduction of N. Nitrous oxide may also be produced by denitrification where nitrite (NO_2^-) may be reduced to NO, N_2O, or N_2 instead of being oxidized to NO_3^- (Sordi et al., 2014). Nitrogen excretion contributes to environmental pollution via NH_3 or N_2O volatilization from the soil surface or NO_3 in the soil that may be leached into ground water (Lagrange et al., 2020). Tannins were also described in the past as anti-nutritional factors due to their negative impact on animal nutrition such as lower feed intake, protein, dry matter and N digestibility, LW gains, milk yield, and wool growth (Mueller-Harvey, 2006). However, PEG can bind to tannins and break the already formed tannin–protein complexes as their affinity for tannins is higher than for proteins (Makkar, 2003b). Chapter 3, on low quality feeds with 9.6% CP content, showed that

the addition of PEG in a diet containing 22% *Desmanthus* (JCU1 and JCU4) on a DM basis harvested at the end of the dry season did not have an impact on total VFA, daily LW gain, or DMI, but increased rumen NH_3-N and iso-acid concentrations. As the anti-nutritional effects of phenolic compounds and tannins vary between species and seasons (Mekuriaw et al., 2020), it was considered important to evaluate *Desmanthus* cultivars at a higher dietary inclusion rate and harvested in the late wet season. The addition of PEG (160 g/kg *Desmanthus* DM) was to determine if tannins were affecting N utilisation.

Therefore, the primary objective of this investigation was to evaluate the impact of supplementing Brangus steers on a basal diet of Rhodes grass with either the tropical legume *Desmanthus spp.* or the temperate legume lucerne on animal performance (DMI, LW gain), rumen VFA and plasma metabolite profiles, N intake, rumen NH_3-N, blood urea, faecal N, and urinary N concentrations. This research tested the hypothesis that *tropical steers supplemented with Desmanthus and lucerne legumes would elicit similar responses in plasma metabolite profiles, productive performance, and volatile fatty acids*.

4.2 Materials and Methods

This study was conducted at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Lansdown Research Station, Queensland, Australia (19.59°S, 146.84°E). All procedures complied with the Australian Code for the Care and Use of Animals for Scientific Purposes (Eighth edition, 2013) as approved by the CSIRO Queensland Animal Ethics Committee (Permit Number 2019-32).

4.2.1. Animals and Treatment

Sixteen yearling Brangus steers were fed a basal diet of Rhodes hay supplemented with forage legumes comprising lucerne or one of the following three *Desmanthus* cultivars: *D. virgatus* cv. JCU2, *D. bicornutus* cv. JCU4, and *D. leptophyllus* cv. JCU7. A completely randomized block experimental design was utilized. Steers had an average LW of 232 ± 6 kg and were randomly allocated into four blocks by weight. One block was allocated to lucerne throughout the study, while the remaining three blocks were allocated to a different *Desmanthus* cultivar in each of the three periods as depicted in Figure 4.1. This design was chosen to avoid the expected nutritional perturbations associated with

changing from lucerne to *Desmanthus*. The animals were fed in individual pens. The first 28 days of the trial constituted a background period where all the animals were fed Rhodes grass (*Chloris gayana*) hay only. The background period was followed by a period of 28 days including 10 days to allow the animals to adapt to the Rhodes grass plus lucerne hay or one of the *Desmanthus* cultivars at a planned level of inclusion of 30% DM. Samples of each *Desmanthus* cultivar and lucerne were sent to Feed Central (Charlton, QLD, Australia) for NIRS analysis to match the targeted CP of 21%. Thus, the overall lucerne content of the diet was 21.3% on a DM basis. The following periods lasted 14 days to allow the animals on *Desmanthus* to adapt to the new *Desmanthus* species. In every period, each steer on *Desmanthus* received a different *Desmanthus* cultivar. At the conclusion of the study, animals remained on their respective diets for a further 21 days with half of the animals on each treatment supplemented with polyethylene glycol (PEG 4000, Redox Pty Ltd, Minto, NSW, Australia) at 160 g/kg DM of *Desmanthus* or lucerne to nullify the bioactivity of tannins. This period included a 5-day adaptation period where the animals were fed an increasing level of PEG (50 g/day) before reaching their full consumption amounts. The adaptation period to the diet was considered adequate as it was within the 10 to 14 days range suggested by Cochran and Galyean (1994). All animals were fed ad libitum to 10% refusals over the first seven days of each period. Thereafter, intake was reduced to 90% of *ad libitum*. The three *Desmanthus* cultivars were harvested fresh using a crop chopper (New Holland Model 38 Crop-Chopper®, Haryana, India) on alternate days from a farm located 20 min away from the research station (19°63′ S, 146°90′ E). The fresh *Desmanthus* was consistently harvested at 7:00 am between four- and six-week regrowth to capture the vegetative stage of maturity to minimize differences in nutritive value between the cultivars. The *Desmanthus* was stored at 5 °C in a cool room prior to being fed out. The *Desmanthus* was mixed with chopped Rhodes grass hay (Roto grind model 760, Burrows Enterprises, LLC, Greeley, CO, USA) before being fed to the steers. The lucerne hay was also chopped (Roto grind model 760, Burrows Enterprises, LLC, Greeley, CO, USA) and mixed with Rhodes grass at the same time as the *Desmanthus* treatments immediately before feeding. *Desmanthus*, Rhodes grass and lucerne hay were all cut to a length of 10 cm. Diets were fed out once daily between 9:30–10:00 am and all experimental steers had continuous access to reticulated water and mineral blocks (Trace element Northern, Olsson's, Yennora, NSW, Australia).

It can be noted that one animal on the JCU7 diet was sick at the end of Period 2 and another one on the JCU4 diet injured himself at the end of Period 2 and was unable to recover. Consequently, for JCU7 diet, only data from 10 animals were analysed and for JCU2 and JCU4 diets, 11 animals were analysed. In period 5, only one animal on the JCU7 diet had no PEG.

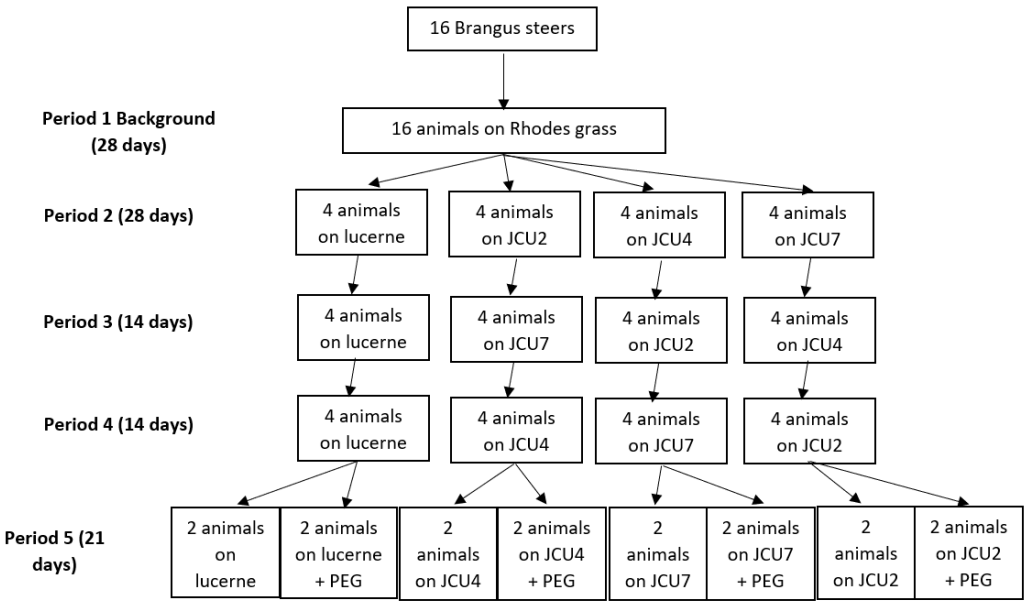


Figure 4.1. Diagram of the experimental design

4.2.2. Feed Chemical Composition and Analysis

Samples of offered (individual forages) and uneaten feeds were taken daily in three consecutive days of the end of each period. Samples were sent for near-infrared reflectance spectroscopy (NIRS) analysis using a scanning monochromator (model 6500, NIRSystem, Inc., Silver Spring, MD, USA) and calibration equations developed by CSIRO Agriculture (Coates and Dixon, 2011) using ISI Software (Infrasoft International, Port Matilda, PA, USA) as described by Durmic et al. (2017). Samples of Rhodes grass, lucerne, JCU2, JCU4, and JCU7 were also sent to FeedTest (Agrifood Technology, Victoria, Australia) for wet chemistry analyses in order to compare the NIRS and wet chemistry results. Due to financial and time constraints, only one sample of Rhodes grass hay, lucerne hay, JCU2, JCU4 and JCU7 were analysed by wet chemistry whereas 20 samples of Rhodes grass hay and 16 samples of lucerne hay, JCU2, JCU4 and JCU7 were analysed by NIRS. The NIRS method giving only a prediction of the feed chemical composition, a comparison between the two methods was necessary to have an idea of the accuracy of the NIRS calibration to predict the feed composition of *Desmanthus* in particular.

The DM (method 934.01) and CP (method 954.01) contents of the samples were determined according to the procedures of the Association of Official Analytical Chemist (2000) (AOAC, 2000). The heat-stable α-amylase-treated NDF and ADF contents were analysed according to the procedure described by Van Soest (1994). *In vitro* DMD was determined using a modified pepsin-cellulase technique described by Clarke et al. (1982).

Metabolizable energy was determined as DMD × 0.172 - 1.707 (CSIRO, 2007) from the NIRS data.

4.2.3. Dry Matter Intake and Liveweight Gain

The LW of each animal was recorded automatically (Gallagher Smart TSI, Melbourne, Victoria, Australia) weekly prior to feeding to determine the daily LW gain. Individual DMI was determined by the difference between offered and residual feed after 24 h. Individual daily intakes were recorded throughout the study to determine treatment group DMI. These values were used to calculate the DMI expressed as % of LW. Feed conversion ratio was calculated as the average of DMI for the 3 periods (periods 2, 3 and 4) on legumes without PEG supplementation divided by the daily LW gain during the same periods.

4.2.4. F.NIRS (faecal NIRS) Estimates of Diet Quality and Estimation of Urinary N

Faecal samples were collected from the rectum of each steer 3 h post-feeding at the end of each period. The samples were dried in a fan- forced oven at 60 °C and ground through a Tecator Cyclotec 1093 (FOSS, Hillerød, North Zealand, Denmark) fitted with a 1-mm screen. A monochromator fitted with a spinning cup module (NIRSystems FOSS 6500, Hilleroed, Denmark) was used to scan faecal NIR spectra at the CSIRO Floreat laboratory (Floreat, WA, Australia). All spectra analyses, data manipulation and spectra calibrations were done with ISI software. The calibration equation for predicting the dietary CP concentration and dry matter digestibility (DMD) (R^2 = 0.92) published by Coates (2004); Coates and Dixon (2007); Coates and Dixon (2011) were used to estimate diet quality. Urinary N concentration was estimated using the equation from Kohn et al. (2005) as follows: Urinary N (g/day) = CR × BUN × LW with CR representing the clearance rate (liters of blood cleared completely

of urea per day which is estimated to be equal to 1.3 for cattle), BUN = blood urea nitrogen (g/L) which is equal to plasma urea divided by 357.1 and LW = liveweight (kg).

Dry matter excretion was considered to be similar between treatments in order to compare N excretion from faeces and urine.

4.2.5. Rumen Collection and Volatile Fatty Acids Analysis

Rumen fluid was collected three hours after feeding on the last day of each period. Rumen fluid samples were collected through an oral stomach tube using a reinforced plastic suction tube (approximately 3 cm in diameter). A hand pump was used to extract 100-200 mL of rumen fluid from the ventral sac. Rumen fluid pH was immediately measured using a pH meter and a sub-sample taken, mixed with fresh 20% metaphosphoric acid (4:1) and frozen at -80 °C for VFA and NH_3-N analyses. Rumen fluid concentrations of short chain fatty acids (acetate, propionate, n-butyrate, iso-butyrate, iso-valerate, n-valerate and n-caproate) were measured by gas chromatography as described by Gagen et al. (2014). NH_3-N concentration was determined by the colorimetric method of Chaney and Marbach (1962).

4.2.6. Blood Sample Collection and Plasma Metabolite Analysis

Blood samples (10 ml) were collected from each experimental steer 3 h after morning feeding at the end of each period. All samples were collected using jugular venipuncture. These were stored in BD Vacutainer® Lithium Heparin Tubes (Becton, Dickinson and Company, Belliver Way, Belliver Industrial Estate, Plymouth, Devon, UK), immediately chilled in an ice-containing esky and later centrifuged at 2500 rpm for 20 min at 4 °C (Allegra® 6 Series and Spinchron™ R Centrifuges, Beckman Coulter, Inc., Brea, California, USA). The plasma was separated from the serum and sub-samples of the plasma were taken and stored at -80 °C until analysis. All samples were analysed for hematological metabolite concentrations at the Veterinary Clinical Pathology Laboratory of the College of Public Health, Medical and Veterinary Sciences at James Cook University, Townsville, Queensland, Australia. Plasma glucose was analysed by an enzymatic UV test (hexokinase method), plasma urea was analysed by kinetic UV test and non-esterified fatty acids (NEFA) were measured with the FA115 kit of Randox (Randox Laboratories Ltd., Crumlin, County Antrim, UK). The three analyses were done on Beckman Coulter AU480 Analyzer (Beckman Coulter, Inc., Brea, California, USA). Cortisol was analysed using

Immulite 1000 Systems analyser (Siemens, Germany) and a solid-phase, competitive chemiluminescent enzyme immunoassay.

4.2.7. Statistical Analyses

All data were analysed using R (Rstudio version 1.3.1056, R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, URL http://www.R-project.org/) with the 'dplyr' (Wickham et al.), 'nlme' (Pinheiro et al., 2021), 'lme4' (Bates et al., 2015), 'car' (Fox and Weisberg, 2019), and 'multcomp' (Hothorn et al., 2008) packages. Effects were considered significant at $p < 0.05$ and considered as "tendency" at $p < 0.06$.

A linear mixed model procedure was utilized to compare the chemical compositions and their effects on intake, daily LW gain, rumen VFA and plasma metabolite profiles between the four different treatments (lucerne, JCU2, JCU4 and JCU7 diet). DMI, LW, daily LW gain, DMI/kg LW, feed conversion ratio, rumen VFA and plasma metabolites, dietary N, N intake, rumen NH_3-N, plasma urea, faecal and urinary N were the dependent variables, while the four treatments were the fixed effects and individual animals nested within blocks were the random effects.

$$Y_{ijkl} = \mu + A_i(l) + T_j + P_k + B_l + \xi_{ijkl}$$

where *Yijkl* is the response variable of the *i*th animal (i = 1 to 16) nested in the *l*th block (l = 1 to 4) that received the *j*th treatment (j = lucerne, JCU2, JCU4 and JCU7) during the *k*th period (k = 1 to 4), μ is the overall mean of all observations, $A_i(l)$ is the random effect of the experimental animal nested in the *l*th block, T_j is the fixed effect of the treatment, P_k is the fixed effect of the period, B_l is the fixed effect of the block, and ξ_{ijk} is the random error component.

The same model was used to analyse the effect of PEG on intake, rumen VFA, plasma metabolite profiles and N concentrations except that only the data from the animals on the *Desmanthus* diet in period 5 were analysed and the fixed effect was the presence or absence of PEG, hence the use of 12 animals in period 5 statistical analysis. The models were based on the restricted maximum likelihood (REML) technique. Pearson's product–moment correlation analysis was also conducted to estimate correlations and *p*-values between NIRS and wet chemistry determined nutritive values for the forage

samples. Crude protein, ADF, NDF, DMD, and ME were the dependent variables and the analytic method the fixed effect

4.3. Results

4.3.1. Chemical Composition

The nutrient compositions of Rhodes grass hay, lucerne hay, and the three *Desmanthus* cultivars are given in Table 4.1. Rhodes grass had a lower CP concentration and a higher fibre content than the three *Desmanthus* cultivars and lucerne. However, the Rhodes grass DMD was similar to the 3 *Desmanthus* cultivars which had a similar ME between Rhodes grass and the *Desmanthus spp.* JCU2 and JCU7 had similar compositions. Lucerne had a higher CP and lower fibre concentration than the three *Desmanthus* cultivars which resulted in a higher DMD and ME. The NIRS and wet chemistry CP, NDF, DMD, and ME values were highly correlated (Table 4.2). However, the NIRS and wet chemistry values were not significantly different in CP, ADF, DMD, and ME concentrations.

The nutritive values of lucerne and *Desmanthus* treatments are given in Table 4.3. CP contents were similar between treatments. The higher ADF content in the *Desmanthus* cultivars compared to lucerne induced a significantly lower ME in the *Desmanthus* diets.

Table 4.1. Chemical compositions (mean ± s.e.) of dietary components predicted by near infrared reflectance spectroscopy.

Variable	Rhodes grass (*n*=20)	Lucerne (*n*=16)	JCU2 (*D. virgatus*) (*n*=16)	JCU4 (*D. bicornutus*) (*n*=16)	JCU7 (*D. leptophyllus*) (*n*=16)	*Desmanthus spp.*[2] (*n*=48)
DM (%)	84.0 ± 0.94	84.0 ± 1.16	32.3 ± 1.61	30.7 ± 1.26	34.2 ± 0.93	32.3 ± 0.78
CP (% DM)	8.8 ± 0.19	15.1 ± 0.61	10.3 ± 0.99	13.0 ± 0.85	10.6 ± 0.80	11.3 ± 3.65
ADF (% DM)	42.8 ± 0.43	37.5 ± 0.73	44.5 ± 1.33	40.4 ± 1.10	43.4 ± 1.00	42.8 ± 0.70
NDF (% DM)	73.8 ± 0.41	49.6 ± 0.79	57.5 ± 1.38	53.1 ± 1.34	58.5 ± 1.20	56.4 ± 0.81
DMD (%)	50.6 ± 0.56	65.2 ± 1.08	47.8 ± 2.57	51.7 ± 1.55	49.4 ± 1.41	49.6 ± 0.011
ME (MJ/kg DM)[1]	7.0 ± 0.10	9.5 ± 0.19	6.5 ± 0.44	7.2 ± 0.27	6.8 ± 0.24	7.4 ± 0.19

[1]Estimated as DMD × 0.172 – 1.707 (CSIRO, 2007), [2]Average of the three *Desmanthus spp*. DM = dry matter, CP = crude protein, ADF = acid detergent fibre, NDF = neutral detergent fibre, DMD = dry matter digestibility, ME = metabolizable energy.

Table 4.2. Comparative chemical composition of the experimental dietary component determined by wet chemistry and NIRS.

Variable	Rhodes grass (*n*=1)		Lucerne (*n*=1)		JCU2 (*D. virgatus*) (*n*=1)		JCU4 (*D. bicornutus*) (*n*=1)		JCU7 (*D. leptophyllus*) (*n*=1)		*Desmanthus spp.*[2] (*n*=3)	r between NIR and wet chemistry values	*p*-Value
	Wet chemistry	NIRS	Wet chemistry	NIRS	Wet chemistry	NIRS	Wet chemistry	NIRS	Wet chemistry	NIRS			
CP (% DM)	9.8	9.0	17.0	16.6	19.6	13.8	17.4	16.7	14.3	7.5	17.1	0.71	0.18
ADF (% DM)	40.0	44.0	35.3	36.3	21.9	39.7	30.9	35.9	30.5	46.7	27.8	0.14	0.82
NDF (% DM)	72.7	74.7	47.4	48.5	33.0	47.3	43.5	49.8	45.8	61.8	40.8	0.89	0.04
DMD (%)	45.3	48.4	58.8	70.0	50.1	60.6	41.8	59.0	43.2	48.3	45.1	0.79	0.11
ME (MJ/kg DM)[1]	6.1	6.6	8.4	10.3	6.9	8.7	5.5	8.4	5.8	6.6	7.9	0.79	0.11

[1]Estimated as DMD × 0.172 – 1.707 (CSIRO, 2007), [2]Average of the three *Desmanthus spp.* calculated using the wet chemistry results. DM = dry matter, CP = crude protein, ADF = acid detergent fibre, NDF = neutral detergent fibre, DMD = dry matter digestibility, ME = metabolizable energy.

Table 4.3. Nutritive value (mean ± s.e.) of diets containing lucerne and *Desmanthus* spp. cultivars determined by NIRS.

Variable	Lucerne (*n*=16)	JCU2 (*n*=16)	JCU4 (*n*=16)	JCU7 (*n*=16)	*Desmanthus spp.*[2] (*n*=55)	SEM	*p*-Value
CP (% DM)	10.2	9.2	10.1	9.4	9.6	0.17	0.08
ADF (% DM)	41.3[a]	41.9[b]	41.9[b]	42.8[b]	42.6	0.62	0.01
NDF (% DM)	68.4	68.7	67.2	68.8	68.3	0.74	0.13
ME (MJ/kg DM)[1]	7.6[a]	7.1[b]	7.1[b]	7.0[b]	7.0	0.06	0.01

[1]Estimated as DMD × 0.172 – 1.707 (CSIRO, 2007), [2]Average of the three *Desmanthus spp*. DM = dry matter, CP = crude protein, ADF = acid detergent fibre, NDF = neutral detergent fibre, ME = metabolizable energy, SEM = standard error of the mean. Means within the same row without the same alphabetical characters (a, b) represent statistical differences ($p < 0.05$).

4.3.2. Animal Performance

As the animals changed *Desmanthus* cultivars in each period to minimize variability, the animal performance was inferred from the average of the 3 *Desmanthus* spp. As shown in Table 4.4, at the start of the experiment, the DMI and LW of all animals were similar. At the end of the feeding trial, the animals fed lucerne had significantly higher DMI and LW than the animals fed *Desmanthus* spp. Therefore, the animals on the lucerne diet had a significantly higher daily LW gain than the animals on *Desmanthus* spp. diet. When the DMI was expressed as DMI/kg LW percentage, it was the same for animals fed lucerne and *Desmanthus spp*. Although the feed conversion ratio (FCR) of the animals on the *Desmanthus* spp. diet was numerically higher than the FCR of the animals on the lucerne diet, it was not significantly different.

Table 4.4. Initial and final DMI, LW, daily gains and feed conversion ratio of steers fed lucerne and *Desmanthus* spp.

Variable	Lucerne (*n*=12)	*Desmanthus spp.* (*n*=32)	SEM	*p*-Value
Initial DMI (kg/day)	5.5	5.4	0.12	0.67
Final DMI (kg/day)	6.5	6.1	0.14	0.03
Initial LW (kg)	270.7	275.0	0.04	0.95
Final LW (kg)	320.0	303.4	0.03	0.04
Daily LW gain (kg/day)	0.6	0.34	0.04	0.01
DMI/kg LW (%)	2.0	2.0	0.03	0.98
Feed conversion ratio	10.6	22.9	4.70	0.19

DMI = dry matter intake, LW = liveweight, SEM = standard error of the mean.

4.3.3. Effect of Lucerne and Desmanthus on Rumen and Plasma Metabolites

The concentration of iso-valerate and NEFA were significantly higher in animals fed lucerne compared to the animals on the JCU4 diet (Table 4.5). Total VFA concentration was significantly higher for the animals on lucerne diet compared to the ones on JCU7 and the concentration of iso-butyrate was significantly higher for the animals on lucerne compared to the ones on JCU4 and JCU7. There was no difference in acetate, propionate, acetate:propionate ratio, n-butyrate, n-valerate, n-caproate, glucose and cortisol between the animals fed lucerne or *Desmanthus* spp. The pH was similar regardless of the type of legume supplement.

Table 4.5. Rumen VFA and plasma metabolites of steers fed lucerne and *Desmanthus* spp.

Variable	Lucerne (*n*=12)	JCU2 (*n*=11)	JCU4 (*n*=11)	JCU7 (*n*=10)	*Desmanthus* spp.[1] (*n*=32)	SEM	*p-V*alue
Rumen volatile fatty acids							
Total VFA (mM)	65.2^{a}	60.2^{ab}	57.0^{ab}	51.5^{b}	56.4	1.64	0.01
Acetate (molar %)	75.4	76.5	75.9	76.8	76.4	0.27	0.17
Propionate (molar %)	14.5	13.9	14.5	14.0	14.2	0.15	0.34
Acetate/propionate ratio	5.2	5.5	5.3	5.5	5.4	0.08	0.34
Iso-Butyrate (molar %)	0.97^{a}	0.81^{ab}	0.76^{b}	0.80^{b}	0.79	0.02	0.01
n-Butyrate (molar %)	7.0	6.8	6.9	6.6	6.8	0.13	0.63
Iso-Valerate (molar %)	1.0^{a}	0.87^{ab}	0.83^{b}	0.86^{ab}	0.85	0.02	0.02
n-Valerate (molar %)	0.95	0.89	0.93	0.81	0.88	0.04	0.65
n-Caproate (molar %)	0.15	0.16	0.17	0.17	0.17	0.01	0.87
pH	7.0	7.0	7.1	7.2	7.1	0.03	0.10
Plasma metabolites							
Glucose (mmol/L)	4.2	4.1	4.2	4.1	4.1	0.05	0.08
NEFA (mmol/L)	0.053^{a}	0.074^{ab}	0.11^{b}	0.081^{ab}	0.088	0.01	0.01
Cortisol (nmol/L)	28.0	30.3	23.7	27.6	24.3	3.39	0.97

[1]Average of the three *Desmanthus* spp. VFA = volatile fatty acids, SEM = standard error of the mean, NEFA = non-esterified fatty acids. Means within the same row without the same alphabetical characters (a,b) represent statistical differences ($p < 0.05$).

4.3.4. Nitrogen Metabolism

As depicted in Table 4.6, dietary N values from wet chemistry analyses were similar in *Desmanthus spp*. and lucerne, while predicted dietary N from F.NIRS analysis was higher in lucerne than in *Desmanthus*, resulting in a significantly higher N intake in animals supplemented with lucerne compared to JCU2 and JCU7 diets. Faecal N concentration was significantly lower in animals fed lucerne compared to those on *Desmanthus*. Between the cultivars, faecal N was significantly higher in animals fed JCU7 compared to those on JCU2 and JCU4. There were no differences in rumen NH_3-N, plasma urea and urinary N between the different diets.

Table 4.6. Dietary, plasma, rumen, faecal and urinary N metabolism in steers supplemented with lucerne and *Desmanthus* spp.

Variable	Lucerne (*n*=12)	JCU2 (*n*=11)	JCU4 (*n*=11)	JCU7 (*n*=10)	*Desmanthus* spp.[3] (*n*=32)	SEM	*p*-Value
Diet N (%DM)[1]	1.6	1.5	1.6	1.5	1.5	0.03	0.30
Diet N by F.NIRS (%DM)	2.4[a]	2.2[b]	2.2[b]	2.2[b]	2.2	0.04	0.01
N intake (g/day)[1]	111.8[a]	92.8[b]	101.7[ab]	92.0[b]	95.8	2.77	0.01
Rumen NH_3-N (mg/dL)	17.6	15.5	16.4	15.6	15.8	0.46	0.32
Plasma urea (mmol/L)	5.3	5.4	5.6	5.5	5.5	0.16	0.96
Faecal N (%DM)	1.8[a]	1.9[b]	2.0[b]	2.1[c]	2.0	0.03	0.01
Urinary N (g/day)[2]	59.3	58.5	59.3	60.0	59.3	1.92	0.64

[1]Estimated by near infrared spectroscopy. [2]Estimated as CR x BUN x LW (with CR = clearance rates of 1.3 for cattle, BUN = blood urea nitrogen, LW = liveweight) (Kohn et al., 2005). [3]Average of the three *Desmanthus spp.* N = nitrogen, SEM = standard error of the mean. Means within the same row without the same alphabetical characters (a, b, c) represent statistical differences ($p < 0.05$).

4.3.5. Effect of Polyethylene Glycol on Animal Performance, Rumen VFA, Plasma Metabolites and Nitrogen Retention

The PEG effect was compared only between the animals fed *Desmanthus* (Table 4.7). The PEG addition had no effect on DMI, plasma metabolites, and N concentrations. Only the concentration of rumen iso-butyrate, iso-valerate, and n-valerate significantly increased with the PEG addition.

Table 4.7. Effect of polyethylene glycol on animal performance, rumen VFA, plasma metabolites and nitrogen concentrations.

Variable	*Desmanthus spp.*		SEM	*p-V*alue
	No PEG (*n*=5)	PEG (*n*=6)		
Animal performance				
DMI (kg/day)	5.6	6.2	0.21	0.20
Rumen volatile fatty acids				
Total VFA (mM)	37.7	40.9	3.60	0.69
Acetate (molar %)	80.1	77.9	0.50	0.06
Propionate (molar %)	12.2	13.0	0.28	0.23
Acetate/propionate ratio	6.6	6.0	0.18	0.17
Iso-Butyrate (molar %)	0.63	0.92	0.05	0.01
n-Butyrate (molar %)	5.4	6.0	0.16	0.18
Iso-Valerate (molar %)	0.7	1.0	0.06	0.01
n-Valerate (molar %)	0.84	0.98	0.04	0.04
n-Caproate (molar %)	0.14	0.16	0.01	0.54
pH	7.0	7.3	0.08	0.11
Plasma metabolites				
Glucose (mmol/L)	4.2	4.4	0.11	0.43
NEFA (mmol/L)	0.12	0.12	0.02	0.99
Cortisol (nmol/L)	25.9	22.2	5.12	0.73
Nitrogen concentrations				
Diet N (%DM)[1]	1.5	1.6	0.05	0.36
Diet N by F.NIRS (%DM)	2.1	2.2	0.05	0.98
N intake (g/day)[1]	99.3	108.0	6.48	0.51
Rumen NH_3-N (mg/dL)	15.6	15.5	1.27	0.95
Plasma urea (mmol/L)	5.6	6.1	0.28	0.43
Faecal N (%DM)	2.1	2.2	0.05	0.05
Urinary N (g/day)[2]	62.6	73.4	4.36	0.35

[1]Estimated by near infrared spectroscopy. [2]Estimated as CR x BUN x LW (with CR = clearance rates of 1.3 for cattle, BUN = blood urea nitrogen) (Kohn et al., 2005). PEG = polyethylene glycol, SEM = standard error of the mean, DMI = dry matter intake, N = nitrogen, VFA = volatile fatty acids, NEFA = non-esterified fatty acids.

4.4. Discussion

The objective of this study was to compare three species of *Desmanthus*, a tropically adapted legume, with the well characterized and widely grown temperate legume, lucerne. Across most indices measured in this paper, the results for lucerne are consistent with the literature (Kanani et al., 2006; Le et al., 2019; McDonald et al., 2003; McDonnell et al., 2017), confirming it to be a legume of high nutritive value. *Desmanthus spp.* were of a lower quality with higher fibre and lower energy content than lucerne which resulted in lower intake and performance. However, it should be noted that lucerne was fed as

hay and was of consistent nutritive value throughout the trial. Securing and feeding a consistent and acceptable supply of *Desmanthus* over three months was challenging and this may have influenced the results. The anticipated temporal variation in chemical composition and nutritive value of *Desmanthus* necessitated the adoption of a randomized block design for the *Desmanthus* treatments, even if this could incur nutritional perturbations as animals shift from one cultivar to another.

4.4.1. Chemical Composition

NIRS predictions for 20 species of perennial legumes showed a good correlation with the wet chemistry results with an $R^2 > 0.7$ for NDF, ADF, DMD, OM, ME and N concentrations (Norman et al., 2020). The lower correlation in the present study for the determination of ADF suggests that more calibration studies are needed to better predict these values for *Desmanthus*. The high correlation between the NIRS and wet chemistry data for NDF content suggests a strong relationship, but this observation should be interpreted with caution given the smaller sample size in this study compared to the 4385 samples analysed by Norman et al. (2020). However, regardless of the method used to analyse the feed composition of the forage, it appeared that lucerne was of a higher quality than the 3 *Desmanthus* cultivars with a higher CP and lower fibre content.

4.4.2. Animal Performance

Chapter 3 demonstrated a daily LW gain of 0.18 kg with 31% *Desmanthus* (JCU1 or JCU4) inclusion in the diet. In my present study, a daily LW gain of 0.34 kg was obtained by supplementing steers with 30% *Desmanthus* on a DM basis. The higher daily LW gain can be explained by the higher quality of the *Desmanthus* and Rhodes grass resulting in higher DMI/kg LW (2% for the current study compared to 1.6% for the previous study). The significantly higher daily LW gain observed in the animals on lucerne compared to *Desmanthus* can be due to the higher feed quality of the lucerne treatment and a higher voluntary consumption of lucerne compared to the *Desmanthus* spp. Kanani et al. (2006) compared intake and growth performance of goats fed Sudangrass supplemented ad libitum with either lucerne or *Desmanthus*, where the nutritive value was similar between the two legumes. Voluntary intake of *Desmanthus* was lower than for lucerne and this was reflected in lower LW gain. Sonawane et al. (2019) replaced a concentrate diet with either 50 or 100 % *D. virgatus* and showed that goats fed

the 50% *Desmanthus* diet had the highest LW gain despite having a lower intake compared with the 100% or 0% *Desmanthus* diets. My results broadly corroborate those of Kanani et al. (2006) and Sonawane et al. (2019) that suggest that performance of animals fed *Desmanthus*-containing diets was lower than animal fed diets containing lucerne or concentrates.

4.4.3. Effect of Lucerne and Desmanthus on Rumen Volatile Fatty Acids and Plasma Metabolites

Volatile fatty acids constitute the main source of metabolizable energy from rumen fermentation in ruminants (Bergman, 1990). In my study, the higher concentration of total VFA in the animals on lucerne compared to the animals fed JCU7 may be due to the significantly greater supply of protein-N which once proteolyzed form amino acids and are deaminated, and VFA produced by fermentation of the carbon skeletons formed (Brandao and Faciola, 2019). The higher ME in the lucerne diet coupled with additional VFA from amino acid catabolism was associated with an additional 260 g/d LW gain compared to cattle fed the *Desmanthus* diets. The difference in VFA concentration may also be associated with the presence of tannins in the *Desmanthus* spp. (Jayanegara et al., 2015; Vandermeulen et al., 2018), although Chapter 3 showed previously that rumen VFA concentration was linearly correlated with an increasing level of *Desmanthus* in the diet. In the present study, the concentration of iso-butyrate was significantly higher in the rumen of animals fed lucerne than those on JCU4 and JCU7, while the concentration of iso-valerate was significantly higher for the animals fed lucerne than those on JCU4. The branched-chain VFA derived from branched-chain amino acids, tend to increase with an increase in dietary N in the rumen (Hristov et al., 2004). Thus, the concentration of iso-acids was higher in animals fed lucerne than in the ones fed JCU4 and JCU7 due to the higher CP intake. This observation corroborates previous findings by Martinez-Fernandez et al. (2020) who reported an increase in rumen iso-acids in cattle supplemented with N compared to the animals fed an un-supplemented diet. The lack of treatment effect on the main VFA proportions (acetate and propionate) suggests that *Desmanthus* spp. and tannins had no negative impact on rumen digestibility. However, total VFA (mM) were affected by the treatment, suggesting an impaired rumen digestibility of Lucerne vs. JCU7 diet. Similar to my observation, Aboagye et al. (2018) and Aguerre et al. (2016) did not find any effect of tannins on propionate and acetate in cattle. This observation was in contrast with a previous report by Beauchemin

et al. (2007b) who found a linear decrease in acetate with an increase in quebracho tannin extract in growing beef cattle fed a forage-based diet with 16.0% CP. Chapter 3 reported a linear increase in acetate to propionate ratio with an increase in *Desmanthus* level in a 9.6% CP diet. This difference may be due to variation in diet quality and processing. The rumen pH in my study was within the range of the normal pH of the rumen fluid in cattle fed a pasture diet (6.0-7.2) (Kiro, 2017). The absence of the effect of tannin treatments on pH corroborates the findings of other studies with tannins in cattle feeding trials (Aboagye et al., 2018) and Chapter 3.

Glucose and NEFA were within the range of normal metabolite concentrations found in cattle (Foroutan et al., 2020b; Grünwaldt et al., 2005; Hammond, 1983; Polkinghorne et al., 2018; Rubio Lozano et al., 2015; Singh et al., 2019). Russel and Wright (1983) stated that plasma glucose concentration was not likely to constitute a useful index of energy intake or status (R^2 = 0.04) in housed or grazing animals due to the insensitivity of circulating concentrations to nutritional change and its concurrent sensitivity to stress (Lindsay, 1978). Clemmons et al. (2017) also showed no difference in glucose concentrations between steers of low and high RFI. They attributed this lack of difference to the tight regulation of glucose in ruminants. On the other hand, plasma NEFA has been shown to be highly correlated with energy intake in the diet (R^2 = 0.89) and consequently a useful index of energy status in animals in different physiological states (Lindsay, 1978; Russel and Wright, 1983). Russel and Wright (1983) found a logarithmic regression relationship between plasma NEFA and energy intake in non-pregnant and non-lactating grazing cattle. In the current trial, NEFA increased in *Desmanthus*-fed cattle (1.66-fold) corresponding to a 14% reduction in ME intake. Cortisol, being a product of the hypothalamic-pituitary-adrenal axis which coordinates physiological stress response, is a biomarker of stress in animals (Llonch et al., 2018). The lack of difference in cortisol between treatments showed that there was no stress due to the legume supplementation.

4.4.4. Nitrogen Concentration in Animals Fed Lucerne and Desmanthus

Dietary protein in excess of animal requirement results in high concentrations of urea in the blood and urine. Urea-N is a fraction of total urinary N. It surges with an increase in dietary protein supply (Dijkstra et al., 2013). Ruminants retain, on average, between 10 to 45% of dietary N as milk or meat,

with the majority excreted in faeces and urine (Aboagye and Beauchemin, 2019; Calsamiglia et al., 2010; Hristov et al., 2019). NH_3 is produced in the rumen and hindgut by microorganisms and the catabolism of amino acids and other N-containing substrates in intermediary metabolism. Urea formation occurs mainly in the liver as a means of detoxification of NH_3 present in systemic circulation. In cattle, net urea-N released by the liver accounts for 65% of increments in N intake (Dijkstra et al., 2013). On average, 47% of hepatic ureagenesis is returned to the gut through the portal-drained viscera (Lapierre et al., 2005).

The lack of difference in rumen NH_3-N between the treatments was likely a consequence of similar CP in the treatments because dietary CP is correlated with NH_3-N concentration (Brandao and Faciola, 2019). Rumen NH_3-N herein was higher than in the previous study in 2018 (8 mg/dL) (Chapter 3), reflecting the increased diet quality in the current trial with higher dietary CP and lower NDF. Plasma urea concentration between 2.86 and 3.57 mmol/L were considered to be an optimal balance between energy intake and digestible protein. A plasma urea concentration exceeding 3.57 mmol/L was indicative of protein wastage (Hammond et al., 1994). The plasma urea concentration in my study was above 3.57 mmol/L, indicating protein wastage excreted in the faeces and urine. The absence of any difference in plasma urea and rumen NH_3-N concentrations reflected the negligible difference in dietary N.

The higher faecal N in animals fed *Desmanthus* spp. than those on lucerne was reflective of the lower N in the diet and the potential effect of tannins. Previous studies had attributed higher faecal N to the presence of tannins as undigested tannin-protein complexes excreted in the faeces (Dixon and Coates, 2009) or to an enhancement in the absorption of essential amino acids from the small intestine resulting in a shift of N excretion from urine to faeces (Waghorn, 2008). Grainger et al. (2009) found a significant reduction in feed N lost to urine from 39%, 26% and 22% by feeding dairy cows with an increasing amount of *Acacia mearnsii* CT in their diet at 0, 0.9% and 1.8% DMI, respectively. A more recent study by Lagrange et al. (2020) showed that heifers grazing tanniferous legumes such as birdsfoot trefoil and sainfoin had lower urinary N concentrations (3.7 and 3.5 g/L) (6.0 g/L), but higher faecal N (34.5 and 35.5 g/kg) compared to the animals on lucerne (6.0 g/L and 30.5 g/kg for urinary and faecal N, respectively). They also showed that combining tanniferous legumes with lucerne improved urinary N

which declined to 2.24 g/L. Faecal N is mainly in the organic form, which is less volatile than urinary N which is subject to nitrification and losses to ground water (leaching) (Grainger et al., 2009). Sordi et al. (2014) stated that the emission factor for faeces (0.15%) was lower than that of urine (0.26%). The urinary N concentration determined with the equation from Kohn et al. (2005) were within the expected range for cattle (between 21 and 264 g/day).

4.4.5. Effect of Polyethylene Glycol on Animal Performance, Rumen VFA, Plasma Metabolites and Nitrogen Concentrations

The addition of PEG to the diet did not affect DMI which corroborates with Chapter 3 which reported no difference in the DMI between the PEG-supplemented and unsupplemented animals on diets containing 22% JCU1 or JCU4. However, it contradicts the study by Landau et al. (2000) which showed that PEG supplementation may alleviate or even totally neutralise the negative effects of CT on DMI by feeding *Aspidosperma quebracho* to Holstein heifers. The decrease in DMI due to the presence of tannins has been attributed to its astringency property which makes the tissue either unpalatable to salivary proteins or by immobilizing enzymes (Kumar and Singh, 1984). Moreover, concentrations of tannins higher than 5% DM may be toxic to animals and induce desquamation and irritation of the intestinal mucosa, liver and kidneys, resulting in lesions, ulcers and even death (Makkar, 2003b). Yisehak et al. (2014) showed a significant increase in DMI by 9, 5, 10 and 6% with the addition of PEG in the diet of Zebu bulls fed 40% DM leaves of tannin-containing plants *Albizia gummifera, Grewia ferruginea, Prunus africana* and *Syzygium guineense*, respectively. These plants contained 85, 55, 76 and 172 g CT/kg DM, respectively. However, due to non-correlation between the efficacy of PEG addition and CT content, the authors suggested an evaluation of other factors that could help predict the efficacy of PEG such as type of tannin or the interaction with other nutrients. Consequently, the lack of difference in DMI with the addition of PEG may be due to the tannin type, different interactions with other nutrients or the tannin concentration in the diet. Furthermore, the results corroborate with Chapter 3 where I showed no difference in rumen VFA concentrations except an increase in iso-acids. The lower iso-acids concentration in the presence of tannins was attributed to the ability of tannins to bind proteins

and protect them from ruminal deamination as iso-acids are derived from amino acids catabolism in the rumen (Bhatta et al., 2009; Fagundes et al., 2020a; Hristov et al., 2004).

A lower faecal N was expected in the present study in the animals supplemented with PEG, but the results showed a tendency for the faecal N to be higher with the PEG addition. It contradicts the findings by Mkhize et al. (2018) that showed a decrease in faecal N with the addition of PEG in the diet of grazing goats compared to when they were supplemented with water or CT. The higher N intake during the PEG period might explain the higher faecal N concentration in the presence of PEG as N excretion by beef cattle is positively correlated with N intake in the diet (Yan et al., 2007).

4.5. Conclusions

The utilisation of N in *Desmanthus* diets differed from that in lucerne. *Desmanthus virgatus* (JCU2), *Desmanthus bicornutus* (JCU4) and *Desmanthus leptophyllus* (JCU7) showed broadly similar results regarding animal performance, plasma metabolite and VFA profiles and N concentrations. The presence of tannins reduced proteolysis in the rumen, as evidenced by lower rumen NH_3-N, and contributed to higher N flow to the lower tract, as evidenced by higher faecal N concentration compared to lucerne. The inclusion of PEG to nullify the tannin effects induced an increase in rumen iso-acids. *Desmanthus* spp. were of a lower quality with higher fibre and lower energy content than lucerne which resulted in lower intake and performance. Nonetheless, the inclusion of *Desmanthus* in diets has the potential to increase performance of tropical beef cattle in northern Australia, possibly due to a better N utilisation attributable to the presence of tannins. These findings could contribute to increased animal production and performance in the drier parts of northern Australia. Further *in vivo* investigation is needed to better understand the impact of tannins in *Desmanthus* on N utilisation and evaluation of outdoor methane emissions in northern Australian beef cattle supplemented with *Desmanthus*.

4.6. Summary

The hypothesis tested was that tropical steers supplemented with the *Desmanthus* legume and lucerne, a widely characterized temperate legume of high nutritive value, would elicit similar responses in plasma metabolite profiles, productive performance, nitrogen retention, and VFA. The tannin-binding compound, PEG-4000, was added to the diets (160 g/kg *Desmanthus* dry matter) with the objective of

further exploring nitrogen (N) utilisation in the animals supplemented with *Desmanthus* relative to lucerne. From February to June 2020, sixteen yearling Brangus steers (average LW of 232 ± 6 kg) were fed a background diet of Rhodes grass (*Chloris gayana*) hay for 28 days, before introducing three *Desmanthus* cultivars (*Desmanthus virgatus* cv. JCU2, *D. bicornutus* cv. JCU4, *D. leptophyllus* cv. JCU7) and lucerne (*Medicago sativa*) at 30% dry matter intake (DMI). Relative to the backgrounding period, all supplemented steers exhibited similar growth performance. Steers supplemented with *Desmanthus* recorded a lower DMI and animal growth performance, but higher faecal N concentration than animals supplemented with lucerne. Among the three *Desmanthus* cultivars, there were no significant differences in N concentrations, VFA, and plasma metabolite profiles. The addition of PEG induced higher rumen iso-acid concentrations and faecal N excretion. However, feeding *Desmanthus* spp. to tropical *Bos indicus* steers could be a valuable means of increasing N utilisation, which is attributable to the presence of tannins, and, consequently, improve animal productive performance. Since supplementation with lucerne resulted in higher liveweight, daily liveweight gains, and overall animal performance than supplementing with *Desmanthus*, the tested hypothesis that both supplements will elicit similar animal performance does not hold and must be rejected. Further *in vivo* investigation is needed to better understand the impact of tannins in *Desmanthus* on N utilisation

Chapter 5 : Evaluation of Methane Emissions in Northern Australian Beef Cattle on a High Quality Diet Supplemented with *Desmanthus* Using Open-circuit Respiration Chambers and Greenfeed Emission Monitoring Systems

5.1. Introduction

The global greenhouse gas emissions from livestock supply chains represent 14.5 percent of all human-induced emissions. Consequently, the livestock sector plays an important part in climate change (Gerber et al., 2013). Enteric methane (CH_4) produced in the gastrointestinal tract of livestock is the single largest source of anthropogenic CH_4 (Knapp et al., 2014). In tropical and subtropical environments such as northern Australia, the poorly digestible pastures with high C:N ratios induce low livestock productivity and increase rumen CH_4 emissions (Fagundes et al., 2020a; Fagundes et al., 2020b). Previous *in vitro* (Durmic et al., 2017; Vandermeulen et al., 2018) and *in vivo* (Chapter 3) studies showed a decrease in CH_4 emissions due to dietary supplementation with *Desmanthus*, a tropical forage legume. *In vitro* studies with *Desmanthus* reported cultivar dependent differences in CH_4 emissions. Durmic et al. (2017) reported lower CH_4 emissions compared to the mean emissions from 23 tropical grasses of 48, 41 and 45% for cultivar JCU1 (*Desmanthus leptophyllus*), cv. JCU2 (*D. virgatus*) and cv. JCU4 (*D. bicornutus*), respectively. Vandermeulen et al. (2018) found significantly higher CH_4 emissions with cv. JCU1 (+33%) and cv. JCU2 (+5%) compared to cv. JCU4. However in the *in vivo* study conducted in Chapter 3, I did not find any significant difference in CH_4 yield between JCU1 and JCU4. Vandermeulen et al. (2018) attributed the *in vitro* reduction in CH_4 emissions to the presence of secondary compounds such as HT, CT and/or their combination in *Desmanthus* spp. Secondary plant compounds such as phenols which include CT and HT, have an important role in feeding strategies to mitigate CH_4 emissions from ruminants (Aboagye and Beauchemin, 2019; Terranova et al., 2020). The results in Chapter 3 showed a positive influence of tannins in *Desmanthus* in which the tannins bind to proteins in the rumen, increase N utilisation and reduce CH_4 emissions. However, the anti-methanogenic property of tannins in *Desmanthus* was inconclusive as the addition of tannin binder PEG

did not affect CH_4 emissions (Chapter 3). The observed reduction in CH_4 emissions was attributed to the positive effect of *Desmanthus* on rumen fermentation as the feed quality increased with an increasing level of *Desmanthus* in the diet. It has been reported that a lower quality diet increases CH_4 emissions (Boadi and Wittenberg, 2002; Gaviria-Uribe et al., 2020). Gaviria-Uribe et al. (2020) reported an inverse relationship between CH_4 yield and DM and OM digestibility. Therefore, a comparative *in vivo* study utilising a non-tannin treatment and another treatment including *Desmanthus* with similar nutritive value would clarify the impact of *Desmanthus* and plant secondary compounds on CH_4 emissions. Furthermore, due to discrepancies in CH_4 emissions between previous *in vitro* and *in vivo* results comparing several *Desmanthus* cultivars, an *in vivo* pen feeding trial with a larger group of animals to test the cultivar effect on CH_4 emissions would also provide further clarity. Lucerne (*Medicago sativa*) is a commonly used temperate perennial legume in southern Queensland and northern New South Wales for its high quality crude protein (Kanani et al., 2006; McDonnell et al., 2017). The comparative impact of supplementing beef cattle on a basal diet of Rhodes grass with varying levels of the tropical forage legume *Desmanthus* spp. and the temperate legume lucerne on CH_4 emissions may fill in this significant knowledge gap in tropical beef cattle nutrition.

Open-circuit respiration chambers are considered as the "gold standard" for accurately measuring CH_4 production from rumen and hindgut fermentation (Charmley et al., 2011; Goopy et al., 2016; Thompson and Rowntree, 2020; Zhao et al., 2020b). However, OC are expensive to construct, technically demanding to operate and maintain, and cannot be used to measure many animals at once (Zhao et al., 2020b). Furthermore, feed intakes are generally lower in OC with the possibility of inducing higher CH_4 yields (expressed as g/kg DMI) (Llonch et al., 2016; Llonch et al., 2018). In contrast, the GreenFeed emission monitoring system is a patented automated head-chambers system based on spot sampling (2-6 min) of eructated and exhaled gases allowing measurements of enteric CH_4 production on a large number of animals under on-farm conditions (Huhtanen et al., 2019). This technique has minimal labor input and interference with animal behavior and production (Waghorn et al., 2016). Previous studies reported minor differences between OC and GEM methods in average CH_4 emission values (Doreau et al., 2018). In this study, the comparative effect of four different dietary inclusion levels of *Desmanthus* on CH_4 emissions was evaluated using GEM in a pen-based experiment imitating

a grazing situation. The overall objectives of this investigation were to compare the antimethanogenic effect of three *Desmanthus* cultivars and the impact of supplementing Brangus steers on a basal diet of Rhodes grass with the tropical forage legume *Desmanthus* spp. and the temperate legume lucerne, on CH_4 emissions. The study tested the hypothesis that *increasing the proportion of Desmanthus in the diet will reduce CH_4 emissions when measured by GEM*.

5.2. Materials and Methods

5.2.1. Experimental Procedures

Two experiments were conducted at the Commonwealth Industrial and Scientific Research Organization (CSIRO) Lansdown Research Station, Queensland, Australia (19.59°S, 146.84°E) following the Australian Code for the Care and use of Animals for Scientific Purposes (Eighth edition, 2013) and were approved by the CSIRO Queensland Animal Ethics Committee. Experiment 1 took place from the 1st of February to the 26th of June 2020 (Permit Number 2019-32) and aimed to compare the effect of supplementing tropical beef cattle with *Desmanthus* cultivars JCU2, JCU4 and JCU7 or lucerne on *in vivo* CH_4 emissions measured by OC. Experiment 2 was carried out from the 17th of March to the 21st of July 2020 (Permit Number 2019-38) and aimed to investigate the effect of incrementally supplementing tropical beef cattle with equal proportion of the three *Desmanthus* cultivars on CH_4 emissions measured by GEM system.

5.2.2. Desmanthus Cultivars

JCU2 (*D. virgatus*) has been selected for its rapid growth, seed set, persistence under grazing and plant density relative to other *Desmanthus* cultivars (Cook et al., 2020; Loch, 2015). The first flowering days from sowing are around 90.8 days (Loch, 2015). This cultivar is reported to perform well across a number of environments with buffel grass and native grasses in northern and central Queensland (Gardiner, 2016). JCU4 (*D. bicornutus*) was selected for its persistence and plant density (Loch, 2015). It is a robust early maturing plant (average of 84 days for the first flowering after sowing) (Gardiner, 2016; Loch, 2015) that is used for pasture improvement on dark clay soils in semi-arid zone (Cook et al., 2020). JCU7 (*D. leptophyllus*) is a late flowering species with limited seed production. It was

selected for its leafiness and bulk production (Cook et al., 2020). Fresh *Desmanthus* was harvested from a farm located 20 min away from the research station.

5.2.3. Experiment 1

5.2.3.1 Animals and Treatments

Experiment 1 is the same experiment as described in Chapter 4. Briefly, a completely randomized block design was used to allocate sixteen yearling Brangus steers weighing 232 ± 6 kg on the average, into four treatments of four animals each based on similar LW. The four treatments comprised Rhodes grass (*Chloris gayana*) as basal diet plus either lucerne (*Medicago sativa*) hay or one of the 3 species of fresh *Desmanthus* (*D. virgatus* (JCU2), *D. bicornutus* (JCU4) and *D. leptophyllus* (JCU7)) at 30% dry matter (DM). The percentage of lucerne in the diet was adjusted periodically to match and equilibrate the crude protein (CP) content in the diets containing *Desmanthus*. Prior to the start of the trial, 16 out of 20 animals were selected based on temperament. These animals were subjected to a three-week training period during which time they adapted to the respiration chambers. There were five periods in this experiment. The first period constituted the backgrounding period where all the animals were offered Rhodes grass for 28 days and adapted to a hay-based diet. The background period was followed by a 28 days duration where the animals were adapted to either lucerne or one of the *Desmanthus* spp. Thereafter, period length was reduced to 14 days as animals were already adapted to legumes in the diet and the cultivar effects on digestion were considered less than introduction of legumes to a grass diet. It should be noted that as fresh *Desmanthus* was being harvested throughout the study, there was an imperative to keep the trial as short as possible to limit nutritional changes over the growing season. During each of the periods 2, 3 and 4, the animals on *Desmanthus* received each *Desmanthus* cultivar once. A final period was included where all the animals from each group stayed on their same *Desmanthus* cultivar diet from period 4 and 2 animals from each group of 4 animals were supplemented with polyethylene glycol (PEG 4000, Redox Pty Ltd, Minto, NSW, Australia) at 160 g/kg *Desmanthus* DM to nullify the bioactivity of tannins. Within each period, animals were fed *ad libitum* (10% uneaten feed after 23 h), then reduced to 90% of *ad libitum* four days before entry into the respiration chambers. The amounts of hay and *Desmanthus* were adjusted daily, weighed out for individual animals and

thoroughly mixed immediately before feeding. The animals were fed once daily between 9:30–10:00 am.

5.2.3.2. Measurement of Methane Emissions

Animals were ranked according to weight and divided into four blocks. Within each block, one animal was allocated at random to one treatment. Four OC were used to measure CH_4 emissions from individual steers as described by Martinez Fernandez et al. (2018) and Chapter 3. Four series of measurements were taken in each period over two weeks. In this way, 16 animals were subjected to chamber measurements over 48 h in four groups of four. Start dates for each period were staggered to ensure all animals were on treatments for the same length of time within each period. Within each series, all four treatments were included (one animal per treatment). Briefly, CH_4 emissions were measured using independent units (23.04 m^3, 3000 L/min airflow) containing drinking water and the daily ration in a feed bin. The internal atmosphere of the chambers was maintained at approximately 24 °C, −10 Pa and relative humidity of 50-75%. Methane production was calculated following a correction of the flow rates to measured conditions for temperature and pressure (Williams et al., 2007). Methane emissions were monitored continuously by infrared absorption (Servomex 4100, Servomex Group Ltd. Crowborough, UK) for 48 h. Methane production (g CH_4/day) was determined using the average of two 24 h measurements. DMI in the chamber was also recorded daily to calculate the CH_4 emissions according to feed intake (CH_4 yield expressed as g/kg DMI).

5.2.4. Experiment 2

5.2.4.1. Animals and Treatments

This experiment was a pen-based feeding trial that ran for 128 days comprising 14 days of adaptation on a basal diet of Rhodes grass hay (9% CP) and the remainder on treatment diets comprising the basal diet and varying levels of *Desmanthus* for 114 days of feeding. Forty-eight animals of 24 – 28 months old in a completely randomized experimental design with an equal number of cattle in four treatment groups were utilized. Cattle were ranked according to weight and blocked into 12 blocks with one animal from each block allocated to one of four treatments (0, 15, 30 or 45% *Desmanthus* inclusion in the diet on DM basis) with four animals per pen and three pens per treatment. Three *Desmanthus*

cultivars (JCU2, JCU4 and JCU7) were fed in equal proportions at each treatment level. The *Desmanthus* treatments were adjusted with lucerne at 7-14 days intervals to obtain a similar CP content in all four treatments. The treatments were 10 cm long and mixed thoroughly daily before feeding. Four cattle from the same treatment were allocated to a pen. Each pen space was 60 m^2 and equipped with a feed bunk, shade and water.

5.2.4.2. Measurement of Methane Emissions

Methane emissions were measured with 4 GEM systems (GEM, C-Lock Inc, Rapid City, South Dakota, USA). Each GEM unit was allocated to the sequential measurement of three pens for 28 days followed by three periods of 10 days (Table 5.1). In each period, one unit was available for 4 animals per pen. Thus, the animals in each pen were monitored by the same GEM unit on two occasions (27 and 10 days duration). The GEM units were solar powered and their operation was initiated when the animal placed its head inside the hood. A radio frequency identification (RFID) reader identified the animal's ear tag which starts the measurement. During visits, enteric gas emissions were measured and pelletized bait feed (Barastoc Calm Performer, Ridley Agriproducts, Harristown, QLD, Australia) was dropped in each session. With the CP concentration of the pellets being around 11%, no pellet effect on the treatments was expected. The details of GEM design, operation and analyses had been described by Hammond et al. (2015). Briefly, an animal puts its head and shoulders into a semi-enclosed space to access feed pellets. Air is drawn past the animal and subsampled for analysis to determine CH_4 concentrations and CO_2 after correction for background concentrations. Only the animal visits of more than 2 min were kept for analysis. The GEM was programmed using C-Lock Inc, Software to deliver a maximum of 4 rotations of a feed dispensing cup delivering approximately 50 g of pellet (as fed) per rotation, with intervals of 45 s between each rotation so that 200 g of pellet was delivered during each visit. A maximum of 5 visits per day (24 h) was allowed with a minimum of 4.8 h required in between visits. The number of drops per animal was recorded and added to the DMI. In total, 2844 GreenFeed visits (an average of 33 visits/ animal and 2.8 visits/day/animal) were collected and processed from this experiment. An average of 36, 26, 35 and 36 visits for the 0, 15, 30 and 45% *Desmanthus* levels respectively, was recorded.

Table 5.1. Order of the pen measured by the four Greenfeed emission monitoring systems

	GEM 1	GEM 2	GEM 3	GEM 4
Period 1 (27 days)	Pen 12 30% *Desmanthus*	Pen 7 45% *Desmanthus*	Pen 1 0% *Desmanthus*	Pen 4 15% *Desmanthus*
Period 2 (27 days)	Pen 10 30% *Desmanthus*	Pen 8 15% *Desmanthus*	Pen 2 0% *Desmanthus*	Pen 5 45% *Desmanthus*
Period 3 (27 days)	Pen 11 0% *Desmanthus*	Pen 9 30% *Desmanthus*	Pen 3 45% *Desmanthus*	Pen 6 15% *Desmanthus*
Period 4 (10 days)	Pen 12 30% *Desmanthus*	Pen 7 45% *Desmanthus*	Pen 1 0% *Desmanthus*	Pen 4 15% *Desmanthus*
Period 5 (10 days)	Pen 10 30% *Desmanthus*	Pen 8 15% *Desmanthus*	Pen 2 0% *Desmanthus*	Pen 5 45% *Desmanthus*
Period 6 (10 days)	Pen 11 0% *Desmanthus*	Pen 9 30% *Desmanthus*	Pen 3 45% *Desmanthus*	Pen 6 15% *Desmanthus*

GEM = GreenFeed emission monitoring

5.2.5. Feed Chemical Composition

The same forages were used in both trials. A scanning monochromator (model 6500, NIRSystem, Inc., Silver Spring, MD, USA) was used to determine the chemical composition of the feed samples by near-infrared reflectance spectroscopy (NIRS) and calibration equations developed by CSIRO Agriculture (Coates and Dixon, 2011) using ISI Software (Infrasoft International, Port Matilda, PA, USA) as described by Durmic et al. (2017).

Metabolizable energy was determined as DMD × 0.172- 1.707 (CSIRO, 2007) from the NIRS data. The CP, ADF, NDF, ME intake were calculated as the CP, ADF, NDF, ME of the dry feed offered minus the CP, ADF, NDF, ME of the dry feed refused after 24 h for Experiment 1 and after one week for Experiment 2.

5.2.6. Plant Extraction Procedure and Analysis of Secondary Compounds

Fresh samples of the three *Desmanthus* cultivars, Rhodes grass and lucerne were sampled every week, stored at −20 °C, freeze-dried at −50 °C for 3 days in a freeze dryer (Epsilon 2-6D LSCplus, Christ, Osterode am Harz, Göttingen, Germany) and ground to pass a 1-mm screen using a Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany) and stored at room temperature (20 °C) (Terrill et al., 1992). A 0.25 mm sieve was used to pass the freeze-dried material before analysis. The laboratory procedure of Terrill et al. (1992) was followed for tannin extraction, except that the supernatant was increased to 300 µL total volume in distilled water.

An estimation of the proanthocyanidin concentration (CT) was determined by the Butanol-HCl-Fe^{III} method using purified *Desmanthus* CT as the standard with absorbance detected at 550 nm (Makkar, 2003a; Porter et al., 1985). Condensed tannin was purified on Sephadex LH-20 as described by Wolfe et al. (2008). The Folin-Ciocalteu method was used to determine the TP concentration with catechin as the standard (Makkar, 2003a).

5.2.7. Dry Matter Intake and Liveweight

In Experiment 1, the LW of each animal was recorded weekly and individual DMI determined by the difference between offered and residual feed after 24 h. In Experiment 2, the LW of each animal was recorded fortnightly and the DMI per pen was calculated by the difference between offered and residual feed after 24 h. The DMI for each animal was calculated by dividing the DMI per pen by four and by adding the weight of pellets eaten. These values were used to calculate the DMI expressed as % LW. CH_4 yield was computed on per kg DMI basis and CH_4 emissions per kg LW.

5.2.8. Statistical Analyses

R (Rstudio version 1.3.1056, R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, URL http://www.R-project.org/) was used to analyse all data with the 'dplyr' (Wickham et al.), 'nlme' (Pinheiro et al., 2021), 'lme4' (Bates et al., 2015), 'car' (Fox and Weisberg, 2019) and 'multcomp' (Hothorn et al., 2008) packages. Effects were considered significant at $p < 0.05$.

In Experiment 1, a linear mixed model procedure was applied to compare the intakes, phenolic concentrations and CH_4 emissions between the three *Desmanthus* spp. (JCU2, JCU4 and JCU7), between lucerne and the three *Desmanthus* spp. and between the background and three *Desmanthus* spp. The intakes, phenolic concentrations and CH_4 emissions were the dependent variables, whilst the treatments were the fixed effects and individual animals nested within blocks were the random effects.

$$Y_{ijklm} = \mu + A_{i(l)} + T_j + P_k + B_l + \xi_{ijklm}$$

where Y_{ijklm} is the m^{th} response variable of the i^{th} animal (i = 1 to 16) nested in the l^{th} block (l = 1 to 4) that received the j^{th} treatment (j = baseline, lucerne, JCU2, JCU4 and JCU7) during the k^{th} period (k = 1 to 4), μ is the overall mean of all observations, $A_{i(l)}$ is the random effect of the experimental animal

nested in the l[th] block, T_j is the fixed effect of the treatment, P_k is the fixed effect of the period, B_l is the fixed effect of the block, and ξ_{ijklm} is the random error component.

The same model was also used to examine the impact of supplementing with PEG on these variables except that only the data from the animals of the *Desmanthus* diet in period 5 were analysed and the fixed effect was the presence or absence of PEG. The model was fitted using the REML procedure.

In Experiment 2, a linear mixed model procedure was utilised to compare the intakes and CH_4 emissions between the 4 treatments. The intakes and CH_4 emissions were the dependent variables, whilst the four treatments were the fixed effects and individual animals nested within pens were the random effects.

$$Y_{ijkl} = \mu + A_i(l) + T_j + P_k + Q_l + \xi_{ijkl}$$

where Y_{ijkl} is the response variable of the ith animal (i = 1 to 48) nested in the lth pen (l = 1 to 12) that received the jth treatment (j = 0, 15, 30, 45% *Desmanthus*) during the kth period (k = 1, 2), μ is the overall mean of all observations, $A_i(l)$ is the random effect of the experimental animal nested in the lth pen, T_j is the fixed effect of the treatment, P_k is the fixed effect of the period, Q_l is the fixed effect of the pen, and ξ_{ijk} is the random error component.

When significant differences were detected, mean separation by pairwise comparison was carried out using the Tukey test.

5.3. Results

5.3.1. Experiment 1

5.3.1.1. Chemical Composition of the Diets

The nutrient and secondary compound intakes in the diet are given in Table 5.1. The intakes of the 3 *Desmanthus* cultivars, CT and TP in the diet were similar. Although the CT in the diet was similar in the three *Desmanthus* diets, Figure 5.1 shows that the CT concentration in JCU4 (5.27 ± 0.43% DM) was significantly higher than in JCU2 (4.10 ± 0.309% DM) and JCU7 (4.13 ± 0.11% DM) (p = 0.014). However, the TP concentration in the 3 *Desmanthus* cultivars was not significantly different (3.32 ± 0.31, 4.14 ± 0.32 and 4.06 ± 0.31% DM as catechin equivalent for JCU2, JCU4 and JCU7, respectively). The TP in JCU2, JCU4 and JCU7 and the CT in JCU2 and JCU4 were not significantly different throughout the trial (Figure 5.1). The CT in JCU7 was significantly higher in period 1 compared to

period 4. The CT and TP in the diet were significantly lower in the background and lucerne diets compared to the *Desmanthus* diets (Table 5.2).

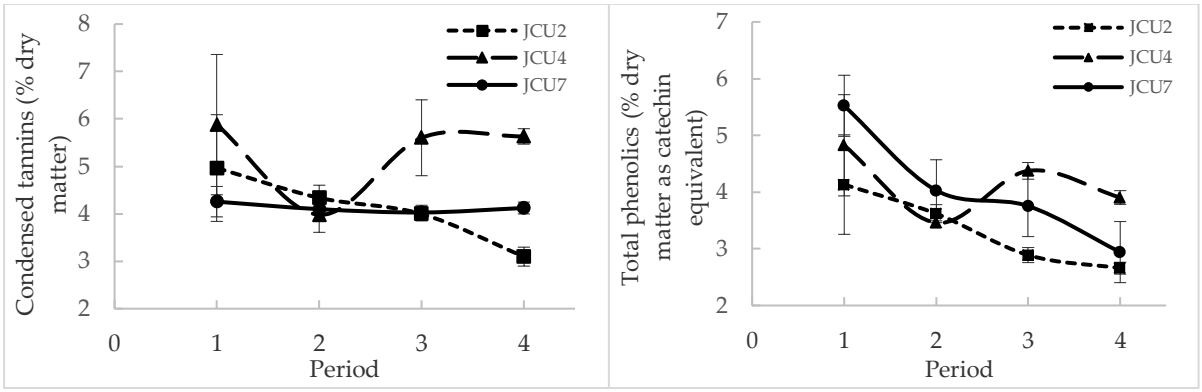
The intakes of DM, CP and ME were significantly lower in the background diet than in the *Desmanthus* diets (Table 5.2). The animals on the lucerne diet had a higher DMI, CP, ADF, NDF and ME intakes than the animals on the 3 *Desmanthus* diets. Although the DMI was higher for the animals on the lucerne diet, the DMI/kg LW was similar for all diets.

It can be noted that the DMI of the animals decreased in the respiration chambers from 5.9 ± 0.09 kg/day to 5.7 ± 0.06 kg/day. However, because all the animals were fed all the *Desmanthus* cultivars, the difference in intake was not taken into account as the aim of the study is to compare the methane emissions between the different treatments.

Table 5.2. Nutrient intakes and phenolic concentrations (± SEM) of the backgrounding, lucerne and *Desmanthus* spp. diets.

Variable	Background (*n*=15)	Lucerne (*n*=12)	JCU2 (*D. virgatus*) (*n*=11)	JCU4 (*D. bicornutus*) (*n*=11)	JCU7 (*D. leptophyllus*) (*n*=10)	*Des. spp.*[1] (*n*=32)	*p*-Value		
							Background vs. *Des spp.*	Luc. Vs. *Des. Spp.*	*Des. Spp.*
DMI (kg/day)	5.47 ± 0.10	6.57 ± 0.10	5.96 ± 0.16	5.85 ± 0.24	5.90 ± 0.21	5.90 ± 0.12	0.01	0.02	0.96
DMI/kg LW (%)	1.99 ± 0.04	2.02 ± 0.07	2.04 ± 0.06	2.03 ± 0.05	1.96 ± 0.04	2.01 ± 0.03	0.70	0.98	0.44
CP intake (kg/day)	0.509 ± 0.01	0.705 ± 0.04	0.568 ± 0.03	0.602 ± 0.02	0.572 ± 0.03	0.582 ± 0.02	0.01	0.01	0.64
ADF intake (kg/day)	2.39 ± 0.04	2.68 ± 0.05	2.51 ± 0.06	2.40 ± 0.11	2.48 ± 0.09	2.46 ± 0.05	0.30	0.02	0.66
NDF intake (kg/day)	4.06 ± 0.08	4.37 ± 0.07	4.05 ± 0.10	3.90 ± 0.16	4.02 ± 0.13	3.99 ± 0.08	0.25	0.01	0.68
ME intake (MJ)	38.3 ± 1.24	52.1 ± 1.23	42.5 ± 1.40	43.1 ± 1.79	42.6 ± 1.62	42.8 ± 0.91	0.01	0.01	0.92
Total phenolics in diet (%DM as catechin equivalent)	0.191	0.313 ± 0.01	1.23 ± 0.10	1.42 ± 0.12	1.50 ± 0.13	1.38 ± 0.07	0.01	0.01	0.21
Condensed tannins in diet (% DM)	0.0799	0.685 ± 0.01	1.44 ± 0.13	1.64 ± 0.21	1.37 ± 0.04	1.49 ± 0.87	0.01	0.01	0.08

[1]Average of the three *Desmanthus spp. Des. Spp. = Desmanthus species*, *Luc.* = lucerne, DMI = dry matter intake, LW = liveweight, CP = crude protein, ADF = acid detergent fibre, NDF = neutral detergent fibre, ME = metabolizable energy, SEM = standard error of the mean.



(5) **(b)**

Figure 5.1. Variation in (a) condensed tannins (% dry matter) and (b) total phenolics (% dry matter as catechin equivalent) throughout the feeding period (*n*=24).

5.3.1.2. Impact of Diet on Methane Emissions

The CH_4 production, yield and CH_4 expressed as g/kg LW were not significantly different between the three *Desmanthus* treatments (Table 5.3). The CH_4 production (g/d) was lower in the background diet and higher in the animals fed lucerne compared to the *Desmanthus* treatments. However, there was no significant difference in the CH_4 yield (g/kg DMI) between all the treatments. The CH_4 expressed as g/kg LW was not significantly different between the background and the *Desmanthus* diets. However, CH_4 expressed as g/kg LW was significantly higher in the animals fed lucerne compared to the animals on the *Desmanthus* diets.

Methane production was highly correlated to DMI ($R^2 = 0.73$) (Figure 5.2). One kg increase in DMI per day increased CH_4 production by 20.99 g/day. There was no correlation between CH_4 yield and CT diet ($p = 0.53$), TP diet ($p = 0.39$), percentage of *Desmanthus* in the diet ($p = 0.96$) or *Desmanthus* DMI ($p = 0.97$).

Table 5.3. Effect of *Desmanthus* spp. on methane emissions.

Variable	Background (*n*=15)	Lucerne (*n*=12)	JCU2 (*D. virgatus*) (*n*=11)	JCU4 (*D. bicornutus*) (*n*=11)	JCU7 (*D. leptophyllus*) (*n*=10)	*Des. spp.*[1] (*n*=32)	SEM	*p*-Value		
								Background vs. *Des spp.*	Luc. vs. *Des. spp.*	*Des. Spp.*
CH_4 production (g/day)	117	137	122	121	123	122	1.93	0.01	0.01	0.92
CH_4 yield (g/kg DMI)	21.6	21.1	21.0	20.8	21.1	21.0	0.21	0.93	0.36	0.93
CH_4 (g/kg LW)	0.428	0.448	0.415	0.414	0.411	0.414	0.01	0.24	0.01	0.83

[1]Average of the three *Desmanthus spp.* DMI = dry matter intake, LW = liveweight, *Des.* = *Desmanthus*, SEM = standard error of the mean.

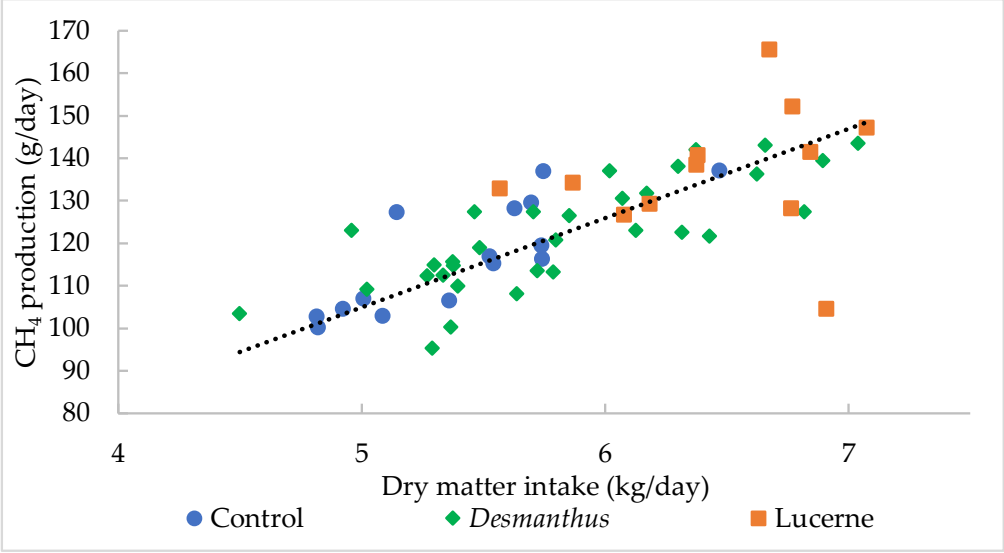


Figure 5.2. The relationship between dry matter intake (kg /day) (including the backgrounding, *Desmanthus* and lucerne diets) and CH_4 production (g/day). The relationship can be described as CH_4 production (g/day) = 20.99x, where x = dry matter intake (kg/day) $R^2 = 0.73$, $p < 0.001$ (n=59).

5.3.1.3. Effect of Polyethylene Glycol on Nutritive Intakes, Phenolics, Tannins Concentrations and Methane Emissions

The addition of PEG to the diet significantly increased the CH_4 production expressed as g/day (Table 5.4). The PEG addition did not have any effect on the intakes and CH_4 yield (g/kg DMI) and expressed as g/kg LW.

Table 5.4. Effect of polyethylene glycol on intakes and methane emissions.

Variable	*Desmanthus* spp.		SEM	*p-V*alue
	No PEG (*n*=5)	PEG (*n*=6)		
DMI (kg/day)	5.6	6.2	0.21	0.20
DMI/kg LW (%)	1.8	1.9	0.04	0.37
CP intake (kg/day)	0.62	0.67	0.04	0.51
ADF intake (kg/day)	2.6	2.6	0.12	0.88
NDF intake (kg/day)	4.3	4.3	0.17	0.78
ME intake (MJ)	44.8	43.6	2.19	0.91
Total phenolics in diet (%DM as catechin equivalent)	1.1	1.1	0.08	0.86
Condensed tannins in diet (% DM)	1.4	1.4	0.12	0.86
CH_4 production (g/day)	125	145	4.16	0.03
CH_4 yield (g/kg DMI)	22.7	23.4	0.56	0.80
CH_4 (g/kg LW)	0.41	0.44	0.01	0.32

PEG = polyethylene glycol, DMI = dry matter intake, CP = crude protein, ADF = acid detergent fibre, NDF = neutral detergent fibre, ME = metabolizable energy, SEM = standard error of the mean, LW = liveweight.

5.3.2. Experiment 2

The effect of level of inclusion of *Desmanthus* cultivars on intake, CH_4 production and yield are presented in Table 5.5. There was no significant difference in the intake of DM (expressed as kg/day or g/kg LW) or CP between the four diets. The ADF intake was significantly lower in the diet containing 45% *Desmanthus* compared to the 0% *Desmanthus* diet. NDF intake was also significantly lower in the 30 and 45% *Desmanthus* diets compared to the 0% *Desmanthus* treatment. ME intake significantly decreased with the increasing level of *Desmanthus* in the diet. Methane production was significantly higher for the 15% and 30% *Desmanthus* inclusion rates compared to the 0% inclusion rate. However, CH_4 production for the 45% inclusion rate was not different to other inclusion levels. The CH_4 yield followed a similar pattern, except that CH_4 yield for the 30% *Desmanthus* inclusion rate was lower than that for the 15% inclusion level. Methane production was correlated to DMI (Figure 5.3). One kg of DMI increase induced an increase in CH_4 production of 23.32 g/day.

Table 5.5. Effect of level of inclusion of *Desmanthus* cultivars on intake, methane production and yield.

Variable	*Desmanthus* in the diet					
	0 (*n*=22)	**15 (*n*=28)**	**30 (*n*=19)**	**45 (*n*=18)**	**SEM**	***p*-Value**
DMI (kg/day)	9.4	8.9	9.0	8.6	2.34	0.16
DMI/kg LW (%)	2.26	2.19	2.23	2.11	0.02	0.77
CP intake (kg/day)	1.05	0.987	0.952	0.927	0.01	0.61
ADF intake (kg/day)	3.51^{a}	3.33^{ab}	3.17^{ab}	3.01^{b}	0.03	0.01
NDF intake (kg/day)	5.78^{a}	5.45^{ab}	5.13^{b}	4.87^{b}	0.06	0.01
ME intake (MJ)	72.7^{a}	69.3^{ab}	65.0^{bc}	62.1^{c}	0.64	0.01
CH_4 production (g/day)	188^{c}	232^{a}	219^{a}	215^{bc}	2.88	0.01
CH_4 yield (g/kg DMI)	20.6^{c}	26.2^{a}	24.2^{b}	25.1^{ab}	0.33	0.01
CH_4 (g/kg LW)	0.450^{b}	0.570^{a}	0.545^{ab}	0.528^{ab}	0.01	0.01

DMI = dry matter intake, CP = crude protein, ADF = acid detergent fibre, NDF = neutral detergent fibre, ME = metabolizable energy, SEM = standard error of the mean, LW = liveweight.

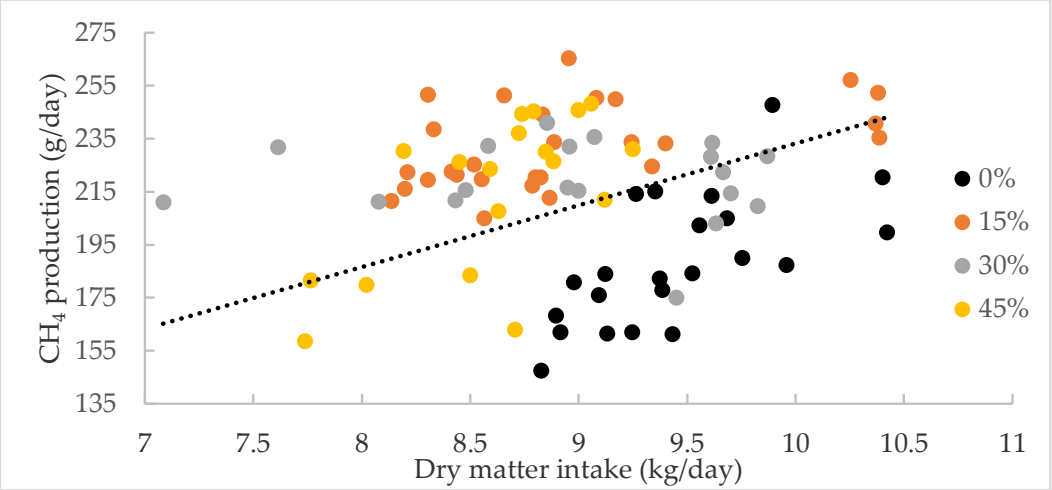


Figure 5.3. The relationship between dry matter intake (kg /day) and CH_4 production (g/day). The relationship can be described as CH_4 production (g/day) = 23.32x, where x = dry matter intake (kg/day) R^2 = 0.68, p = 0.028 (n=87).

5.4. Discussion

5.4.1. Experiment 1

5.4.1.1. Comparison of Methane Emissions Between the *Desmanthus* Cultivars

In general, the chemical composition of *Desmanthus* in the current study suggested lower nutritive value, as evidenced by lower CP and higher NDF compared to the previous *in vitro* studies conducted by Vandermeulen et al. (2018). For instance, they found a CP concentration of 12.6% DM and 13.8% DM for JCU2 and JCU4, respectively, and an NDF concentration of 52.5% DM and 47.8% DM for JCU2 and JCU4, respectively, after 51 days regrowth. The analyses of JCU4 in Chapter 3 showed a CP and NDF concentrations of 14.6 and 58.3 % DM, respectively, after six weeks regrowth. The *Desmanthus* in our present study had a CP concentration of 10.3% DM and 13.0% DM for JCU2 and JCU4, respectively, and a NDF concentration of 57.5% DM and 53.1% DM for JCU2 and JCU4, respectively, after four to six weeks regrowth (Chapter 4). These data collectively demonstrate that the nutritive value of *Desmanthus* can vary widely depending on the cultivar/species, stage of growth, edaphic and climatic conditions. However, JCU2 appears to be of a lower quality than JCU4 (Durmic et al., 2017; Vandermeulen et al., 2018).

The current study showed no significant difference in nutrient intake between the *Desmanthus* diets, presumably due to similar nutritive values. The concentration of TP and CT in the plants in this experiment were higher than the values reported in Chapter 3 where the same standards were used, although the absorbance of the same samples used for the standard curve differed between the two studies, being higher in the current trial. This observation highlights the fact that the TP and CT concentrations can only be compared within a short period of time in the same laboratory (Wolfe et al., 2008). However, the higher CT concentration in JCU4 corroborates with the study conducted by Vandermeulen et al. (2018) where they found a higher CT concentration in JCU4 in winter compared to JCU1 and JCU2 (Table 5.6). They also reported a significantly lower concentration of TP and CT in JCU2 compared to JCU1 and JCU4 in contrast to my current findings. Gonzalez-V et al. (2005) showed an increase in tannin concentration with maturity (from 60 days to 120 days after planting) and a higher tannin concentration in the leaves compared to the stems. The decrease in CT in JCU7 from periods 1 to 4 may be due to a decrease in the leaf to stem ratio or the harvesting of a younger regrowth towards the end of the trial. McMahon et al. (2000) explained the difference in the concentration of CT as a

function of plant maturity due to the activities of four enzymes in the CT biosynthetic pathway which are maximal in young, unexpended leaves of sainfoin and decline or are absent in older leaves. However, the tannin concentration in the plant is also influenced by environmental conditions. For instance, Top et al. (2017) found that the green leaf of *Quercus rubica* produced 50% more tannins when grown in dry conditions compared to wet conditions.

There was no significant difference in CH_4 emissions between the *Desmanthus* cultivars which corroborates the *in vivo* findings in Chapter 3, but contradict the *in vitro* results of Vandermeulen et al. (2018) that showed higher CH_4 emissions expressed as mL/g OM fermented with JCU2 compared to JCU1 and JCU4 after 72h (Table 5.6). On the other hand, Durmic et al. (2017) showed similar *in vitro* CH_4 emissions expressed as mL/g DMI with JCU2 and JCU4 (29.2 and 29.7 mL/g DMI for JCU2 and JCU4, respectively), and lower CH_4 production with JCU1 (24.2 mL/g DMI).

Data from a limited number of studies with these *Desmanthus* cultivars demonstrate the inherent variability in nutritive value, phenolic compounds and methane production both *in vitro* and *in vivo*. While there is evidence to support a relationship between phenolic compound concentration and methane production (Vandermeulen et al., 2018), it is by no means a clear relationship (Aboagye and Beauchemin, 2019). *In vivo* studies require a long feeding period, during which chemical composition may change (Bhatta et al., 2007).

Table 5.6. Comparison of the chemical composition and methane emissions of JCU1, JCU2, JCU4 and JCU7 between the four studies conducted by Durmic et al. (2017), Vandermeulen et al. (2018), the experiment in Chapter 3 and the current study

	JCU1 (*D. leptophyllus*)			**JCU2 (*D. virgatus*)**			**JCU4 (*D. bicornutus*)**				**JCU7 (*D. leptophyllus*)**
	Durmic et al. (2017)	**Vandermeulen et al. (2018)**	**Chapter 3**	**Durmic et al. (2017)**	**Vandermeulen et al. (2018)**	**Current study**	**Durmic et al. (2017)**	**Vandermeulen et al. (2018)**	**Chapter 3**	**Current study**	**Current study**
CP (% DM)	14.5	16.5	11.0	16.0	12.3	10.3	17.5	16.5	14.6	13.0	10.6
NDF (% DM)	45.2	47.8	67.4	50.0	52.2	57.5	48.1	42.6	58.3	53.1	58.5
ADF (% DM)	24.8	25.0	46.3	27.2	31.2	44.5	27.1	20.5	36.8	40.4	43.4
ME (MJ/kg DM)	8.05	-	6.5	8.68	-	6.5	8.56	-	7.3	7.2	6.8
TP	-	8.48% DM as tannic acid equivalent	1.7% DM as catechin equivalent	-	4.25% DM as tannic acid equivalent	3.32% DM as catechin equivalent	-	6.90% DM as tannic acid equivalent	2.3% DM as catechin equivalent	4.14% DM as catechin equivalent	4.06% DM as catechin equivalent
CT	-	3.60% DM as leucocyanidin equivalent	3.5% DM	-	2.09% DM as leucocyanidin equivalent	4.10% DM	-	3.81% DM as leucocyanidin equivalent	3.7% DM	5.27% DM	4.13% DM
CH_4	24.2 mL/g DM	15.4 mL/g OM	16.9 g/kg DMI	29.2 mL/g DM	23.1 mL/g OM	21.0 g/kg DMI	29.7 ml/g DM	21.9 mL/g OM	18.3 g/kg DMI	20.8 g/kg DMI	21.1 g/kg DMI

DM = dry matter, DMI = dry matter intake, CP = crude protein, ADF = acid detergent fibre, NDF = neutral detergent fibre, ME = metabolizable energy, TP = total phenolics, CT = condensed tannins, OM = organic matter.

5.4.1.2. Comparison of Methane Emissions Between the Backgrounding and *Desmanthus* Diets

The lower DMI during the backgrounding period was attributed to the experimental design of the feeding trial as the backgrounding period occurred at the start of the experiment when the animals were smaller. However, the DMI expressed as g/kg LW was not significantly different between the backgrounding and *Desmanthus* diets. The lower DMI expressed as kg/day induced a lower CH_4 production compared to the *Desmanthus* diets as it is correlated to DMI. The current study showed a linear increase in CH_4 production with an increase in DMI with the slope of the equation being 20.99, which corroborates previous findings by Charmley et al. (2016) who reported a slope of 20.7. Benaouda et al. (2020) stated that DMI can explain 78% of the variation in CH_4 emissions and account for up to 92% of the variation when only OC data were used (Charmley et al., 2016). Boadi and Wittenberg (2002) reported a strong correlation ($r = 0.8$) between DMI and CH_4 production with DMI accounting for 64% of daily variation in CH_4 production. Feed intake is therefore one of the key factors accurately accounting for variation in CH_4 emissions in cattle (Benaouda et al., 2020; van Lingen et al., 2019).

The results in Chapter 3 showed an 8% decrease in CH_4 yield (from 19.1 to 17.5g/kg DMI) from 0% to 31% *Desmanthus* inclusion in the diet in contrast to this current study where no significant difference was observed between the background and the *Desmanthus* diets. The presence of tannins in *Desmanthus* is frequently cited as the cause for reduction in methane emissions and was a possible contributory factor, but this will be discussed in more detail in subsequent sections. The lack of difference in CH_4 yield between the background and *Desmanthus* diets could be due to similar NDF intakes in the background and *Desmanthus* diets since NDF intake is directly related with CH_4 emissions (Gaviria-Uribe et al., 2020).

My results contradict the *in vitro* study findings by Vandermeulen et al. (2018) who reported a decrease in CH_4 emissions with the JCU2 and JCU4 *Desmanthus* cultivars compared to Rhodes grass. The difference in CH_4 emissions in their study can be attributed to the higher dietary NDF in Rhodes grass compared to the *Desmanthus* cultivars. In the current study, even though the *Desmanthus* diets were of a higher quality than the backgrounding treatment (higher CP intake) and contained secondary plant compounds (two aspects that would be expected to reduce methane emissions), there was still no difference in CH_4 emissions between the treatments.

5.4.1.3. Comparison of the Methane Emissions Between the Lucerne and *Desmanthus* Diets

There is ample evidence to show that improving the nutritive value of diets reduces methane production (if intake remains unaltered) and methane yield and this holds true for temperate and tropical diets and forage-based and concentrate-based diets. There is also evidence to suggest that bioactive compounds found in tropical legumes, such as *Desmanthus*, can reduce methane production and yield. In the current study, these two drivers of methane production are working in opposing directions. That is, the lower nutritive value of *Desmanthus* versus lucerne may be serving to increase methane production from *Desmanthus* while the bioactive may be serving to reduce methane production from *Desmanthus*. Thus, the overall effect is that there was no difference in methane production or yield between the lucerne and *Desmanthus* treatments.

The lack of significant difference in CH_4 emissions between the lucerne and *Desmanthus* diets in the current *in vivo* study contradicts previous *in vitro* study findings by Durmic et al. (2017) that reported a 27% decrease in CH_4 emissions from *Desmanthus* compared to lucerne. In that study, the lucerne quality was similar to the *Desmanthus*, whereas in the current study the *Desmanthus* cultivars were of lower quality than the lucerne. This might explain the difference the current study and the study conducted by Durmic et al. (2017); the diet quality effect in the current study is negating any possible bioactive effect.

The increase in DMI observed in the lucerne diet can be attributed to the higher digestibility of lucerne (65.2%) compared to *Desmanthus* spp. (49.6%) (Chapter 4). Kennedy and Charmley (2012) reported an increase in both dry organic matter intake (DOMI) and CH_4 production in steers fed lucerne compared to cattle on tropical grasses such as speargrass (*Heteropogon contortus*), buffel grass (*Cenchrus ciliaris*), bisset grass (*Bothriochloa insculpta*), Mitchell grass (*Astrebla lappacea, Astrebla elymoides*), Rhodes grass (*Chloris gayana*) or tropical legumes such as Burgundy bean (*Macroptilium bracteatum*) or Stylo (*Stylosanthes hamata*). They found a decrease in methane emissions expressed as g per kg DOMI by up to 26% and 10% in animals given high quality lucerne compared to the animals given poorer quality buffel grass and Stylo, respectively. The increase in DOMI can be explained by

the higher digestibility of lucerne compared to tropical grasses and legumes which induced a rise in digestive efficiency as reflected by a lesser loss of energy to CH_4 production. Gaviria-Uribe et al. (2020) also showed that methane emissions expressed per kg of DMI and methane intensity expressed per unit liveweight gain were significantly higher in low quality feed composed of Cayman (*Urochloa hybrid*) compared to Cayman mixed with *Leucaena leucocephala* or *Leucaena diversifolia*. Low concentrations of NDF and ADF are characteristic of high-quality forages. Digestible NDF proportions of about 15 and 25 percentage units are optimal for legumes and grasses, respectively, hence, high quality forages are digested quickly, a process which minimizes rumen/gut fill and permits maximum dry matter intake (Benchaar et al., 2001; Mertens, 1994; Popp et al., 2000; Tafaj et al., 2007).

The lower DMI in the *Desmanthus* diets can also be due to lesser palatability compared to lucerne. Palatability is defined as the characteristic of a feed indicating its acceptability regarding gustatory, olfactory or visual senses. It affects an animal's preference for a given feed when offered choice and the rate of eating and intake when offered a single feed (Mertens, 1994). Palatability is often based on astringency associated with CT-protein complexes formed from proteins in saliva. Therefore, the greater the proteins bound by CT, the greater the astringency and the lower the palatability (Naumann et al., 2017). Usually, a depressed intake is seen at dietary CT concentrations exceeding 5% of DM. However, it is possible that intake may be depressed at concentrations less than 5% of DM when the CT are more effective at protein binding and at concentrations greater than 5% DM when the CTs are less effective (Naumann et al., 2017). The *in vivo* study conducted in Chapter 3 showed no decrease in DMI when the animals were supplemented with 31% *Desmanthus*.

The lack of reduction in CH_4 yield in the *Desmanthus* diet can also be explained by the low concentration of tannins (lower than 2% DM for both CT and TP). Previous studies with low or moderate tannin concentrations in the diet failed to reduce enteric CH_4 emissions in cattle. For instance, Beauchemin et al. (2007a) observed a protein-binding effect, but reported no reduction in CH_4 emissions in growing cattle supplemented with 2% DM quebracho tannin extract. Moreover, a recent *in vitro* study conducted by Thirumeignanam et al. (2020) reported a significant decrease in CH_4 production expressed as ml/g/h on a hedge lucerne (*D. virgatus*) silage diet supplemented with 3 and

4% (*w/w*) tannin as tannic acid equivalent from *Acacia nilotica* pods with goats' rumen fluid compared to the diet containing 1, 2 and 5% (*w/w*) tannin as tannic acid.

Therefore, the higher quality and digestibility of lucerne induced a higher nutrient intake compared to the *Desmanthus* treatments. The higher quality of the lucerne diet may have reduced methane yields relative to the lower quality diets that contained *Desmanthus*. This effect could potentially mask a tannin effect on reducing methane emissions from *Desmanthus* cultivars. Thus, two different processes, both acting on methane emissions, may have counteracted one another. The possibility that the secondary plant compounds in the *Desmanthus* were affecting CH_4 emissions cannot be completely ruled out.

5.4.1.4. Effect of Polyethylene Glycol on Methane Emissions

Methane production was significantly higher with PEG addition but no difference in CH_4 yield was observed. This observation corroborates with the results reported in Chapter 3 where no difference in CH_4 yield in the presence of PEG in a diet containing 22% *Desmanthus* DM were detected. However, the results contradict some previous studies where they showed an increase in CH_4 yields expressed as L per kg DMI and mM per g of DM with the addition of PEG in a diet containing tannins at 15% DM (Animut et al., 2008) and 25% DM (Bhatta et al., 2009), respectively. Moreover, Fagundes et al. (2020a) did not find any correlation between CT concentration and biological effect, the biological effect of tannins being an increase in gas production when a binding agent is added (Bueno et al., 2008). They indicated that chemical analysis alone would not predict CT bioactivity which can be related to structure as much as concentration of the molecule. Nevertheless, they found a link between TP content and biological effect. For instance, the species with the greatest biological effect had the highest phenolic content. In the current study, neither CT nor TP was correlated with CH_4 emissions. However, when their biological activity was eliminated with PEG addition, a small elevated CH_4 yield response was observed in the presence of *Desmanthus*. The limited evidence for a tannin effect suggests tannins may have been inhibiting methanogenesis, but the evidence is not strong, possibly due to the low levels of tannins in these *Desmanthus* cultivars. It is important to note that other secondary plant metabolites can contribute to reducing the methane emissions from ruminants, such as saponins, essential oils and flavonoids (Ku-Vera et al., 2020), which might be present in *Desmanthus*.

5.4.2. Experiment 2 - Effect of Level of Inclusion of Desmanthus Cultivars on Intake, Methane Production and Yield

The results of experiment 2 showed no significant difference in DM and CP intakes as lucerne in comparison to Experiment 1 where both intakes were higher in the lucerne diet. These observations are contrary to the report of Chapter 3 with non-isonitrogenous diets that showed a linear increase in DMI with increasing level of *Desmanthus* inclusion in the diet. In contrast, the ADF and NDF intakes decreased with a trend towards lower DMI as *Desmanthus* proportion in the diet increased. Metabolizable energy decreased with increasing level of *Desmanthus* as ME was lower in the *Desmanthus* spp. compared to lucerne. Similarly, the significantly higher CH_4 yields in the 15 and 30% *Desmanthus* treatment compared to the 0% *Desmanthus* (backgrounding) does not align with previous studies either showing a decrease or comparable CH_4 emissions with and without *Desmanthus* (Durmic et al., 2017; Vandermeulen et al., 2018). The difference in CH_4 emissions compared to previous studies might be due to limitations associated with the GreenFeed units. Arbre et al. (2016) stated that to obtain a correlation of r = 0.70 for CH_4 yield (g/kg DMI), a 17-day period for GreenFeed monitoring was necessary along with a number of animals of 6-8 per group to be able to detect a difference of 20% in CH_4 yield among treatments. Manafiazar et al. (2017) also reported that 7 to 14 days with a minimum of 20 samples per animal were necessary to produce repeatable and reliable averaged CH_4 and CO_2 emissions correlated with DMI. In my present study, even though the first periods lasted 28 days, the number of animals per treatment in each period was four. Furthermore, if only the over 20 visits per animal were used, CH_4 yield would have stayed similar (20.2, 26.4, 24.1 and 24.7 for the 0, 15, 30 and 45% *Desmanthus* diets, respectively). Previous studies suggested a lower repeatability when averaged over a long period compared to a shorter monitoring period due to changes in animal physiological status, which can induce between-period variability (Coppa et al., 2021). For instance, Denninger et al. (2019) showed an increase in repeatability of up to 0.68 when the measurement period was extended from 7 to 14 days, but showed a decrease in repeatability when the measurement period was further extended to 28 days. Arthur et al. (2017) reported significantly less heterogeneous variances by taking

the records with a minimum of 3-min GEM visit duration instead of 2-min. In the current study, by taking only the measurements with a minimum of 3-min GEM duration, CH_4 yield was significantly lower in the 0% *Desmanthus* treatment (20.1 g/kg DMI) compared to the three *Desmanthus* treatments (26.8, 25.2 and 24.9 g/kg DMI for the 15, 30 and 45%, respectively). Hristov and Melgar (2020) also reported a need of sufficient number of observations covering the entire 24 h feeding cycle to have representative emission estimates using GEM system, because measures using GEM depended on the time of measurement relative to time of feeding. The increase in CH_4 emissions with an increase in *Desmanthus* level could also be explained by the experimental design as most of the animals in the backgrounding (0% *Desmanthus* DM) treatment were measured by the same unit compared to the other treatments that were measured by two different units. Consequently, comparisons between the GEM units is not feasible.

5.4.3. Comparison Between Open-circuit and GreenFeed Emission Monitoring System

The CH_4 yield measured with the GEM was quantitatively higher compared to OC. However, the slope of the response in methane to DMI (0.0233) was quite similar to Experiment 1 (0.0210). Previous studies showed mixed results regarding the comparison between OC and GEM unit measurements. For instance, Alemu et al. (2017) reported a significantly higher CH_4 yield measured by the GEM system (28.5 g/kg DMI) compared to the OC results (26.5 g/kg DMI) for the same animals. They explained this difference by the decrease in DMI in OC that can vary between 10 to 19% due to the stress associated with change of environment and the decreased energy expenditure in the respiration chamber (Llonch et al., 2016; McGinn et al., 2004). Huhtanen et al. (2019) also reported greater CH_4 production measured by GEM (13 g/day) than those measured by OC. However, Doreau et al. (2018) reported a lower CH_4 emission for GEM than the OC by 14% on average. They attributed this difference to flatus and faeces that are measured in OC and not by the GEM. Although only 2-4% of enteric production is caused by flatulence, they explained the underestimation of CH_4 emissions with GEM due to the missing of the post-prandial peak of emission as the proportion of visits to GEM is low during main meals (Hammond et al., 2016). The correlation between GEM and OC measurements are inconsistent with values fluctuating between 0.37 and 0.32 for dry cows (Doreau et al., 2018), 0.60 and 0.85 for

cattle (Velazco et al., 2016) and between 0.10 and 0.058 for heifers (Hammond et al., 2015). However, Huhtanen et al. (2019) showed a good relationship in CH_4 production measured by OC and GEM (R^2 = 0.92) when they studied 20 direct comparisons. Previous studies generally agreed that minor differences between the two methods for average values, but individual correlations may limit their interchangeability for determining gas emissions of individual animals (Doreau et al., 2018).

5.5 Conclusions

Desmanthus virgatus (JCU2), *D. bicornutus* (JCU4) and *D. leptophyllus* (JCU7) showed no difference in secondary plant compounds concentrations (CT and TP) and in CH_4 emissions. Despite the presence of these compounds in *Desmanthus* spp., no difference was observed in CH_4 yield between the *Desmanthus* treatments, the backgrounding (Rhodes grass) or lucerne diets when CH_4 was measured with OC. The similar CH_4 emissions between the lucerne and *Desmanthus* diets may be attributed to the higher quality and digestibility of lucerne compared to *Desmanthus* and to the low level of secondary plant compounds in the diet. The absence of tannin effect on CH_4 emissions was highlighted with the addition of PEG which did not show any difference.

An increase in CH_4 yield with a *Desmanthus* inclusion level of 15, 30 and 45% DM in the diet was observed when CH_4 was measured with GEM, compared to the Rhodes grass and lucerne treatments. The increase in CH_4 emissions with the addition of *Desmanthus* in the diet might also be due to the higher quality of lucerne and to the possible differences between GEM units. The hypothesis that increasing the proportion of *Desmanthus* in the diet will reduce CH_4 emissions when measured by GEM is rejected. Therefore, on similar high-quality diets, *Desmanthus* does not reduce CH_4 emissions. However, *Desmanthus* can compete with a good quality legume such as lucerne in terms of DM and CP intakes. These findings could contribute to increased intakes in the drier parts of northern Australia where temperate legumes such as lucerne cannot persist. Further *in vivo* investigation is needed to better evaluate the outdoor methane emissions in northern Australian beef cattle supplemented with *Desmanthus*.

5.6. Summary

The main objective of this study was to compare the effect of supplementing beef cattle with *Desmanthus virgatus* cv. JCU2, *D. bicornutus* cv. JCU4 and *D. leptophyllus* cv. JCU7 and lucerne on *in vivo* CH_4 emissions measured by OC or the GEM system. Experiment 1 employed OC and utilized sixteen yearling Brangus steers fed a basal diet of Rhodes grass (*Chloris gayana*) hay in four treatments - the three *Desmanthus* cultivars and lucerne (*Medicago sativa*) at 30% dry matter intake (DMI). Polyethylene glycol was added to the diets to neutralize tannin binding and explore the effect on CH_4 emissions. Experiment 2 employed GEM and utilized forty-eight animals allocated to four treatments including a basal diet of Rhodes grass hay plus the three *Desmanthus* cultivars in equal proportions at 0, 15, 30 and 45% DMI. Lucerne was added to equilibrate crude protein content in all the four treatments. Experiment 1 showed no difference in CH_4 emissions between the *Desmanthus* cultivars, between *Desmanthus* and lucerne or between *Desmanthus* and the basal diet. Experiment 2 showed an increase in CH_4 emissions in the three levels containing *Desmanthus*. It is concluded that on high-quality diets, *Desmanthus* does not reduce CH_4 emissions.

Chapter 6 : General Discussion

Livestock supply chains represent 14.5% of all human-induced greenhouse gas emissions (Gerber et al., 2013). The tropical state of Queensland accounted for 47% of Australia's beef and veal production in 2019, thereby positioning Australia as the second largest beef exporter in the world behind Brazil (MLA, 2020). Enteric methane produced in Queensland represents 3% of Australia's total greenhouse gas emissions (Australian Greenhouse Emissions Information System, 2018). Methane emissions and animal performance are associated with diet quality (Hoffmann et al., 2021). The tropical legume, *Desmanthus*, has shown potential to decrease CH_4 emissions *in vitro* (Durmic et al., 2017; Vandermeulen et al., 2018) and increase animal performance in northern Australia (Collins et al., 2016; Gardiner and Parker, 2012; Ngo, 2017). However, *in vivo* evaluation of animal response to supplementation with *Desmanthus* in terms of CH_4 emission, rumen VFA, plasma metabolites and nitrogen utilisation are fundamental existing knowledge gaps in the northern Australian tropical beef cattle production system.

The studies presented in this thesis tested the overarching hypothesis that supplementing tropical beef cattle steers with *Desmanthus* will improve feed intake, animal growth, N utilisation and reduce in vivo CH_4 emissions without negatively impacting rumen VFA and plasma metabolite profiles. To best investigate this hypothesis, several initial objectives were posed – in the **Introduction** – which gave rise to subordinate hypotheses that contribute to overall comprehension. These studies evaluated the effects of supplementing tropical beef cattle with different *Desmanthus* cultivars and different levels of dietary inclusion and their interactions on:

1) Feed intake;
2) Liveweight gain;
3) Methane emissions;
4) Rumen VFA and plasma metabolites;
5) Nitrogen utilisation.

The daily feed intake and LW gain responses of tropical beef cattle supplemented with different cultivars of *Desmanthus* were assessed in **Chapters 3, 4 and 5**. It was found that overall, *Desmanthus*

cultivar JCU4 was of a higher quality than JCU1, JCU2 and JCU7 in terms of higher CP and lower fibre concentrations in agreement with the *in vitro* results of Vandermeulen et al. (2018) and Durmic et al. (2017) (Chapters 3 and 4). Despite the feed quality difference between the *Desmanthus* cultivars, there was no significant cultivar effect (p>0.05) on DMI and daily LW gain. Detmann et al. (2014) estimated that 10.8% CP in the diet is the minimal CP concentration to have an efficient utilisation of N for an improvement in animal growth. This is higher than the 9.2% CP at 22 and 31% *Desmanthus* inclusion levels in the experimental diet reported in Chapter 3, and 9.4% CP at 30% *Desmanthus* inclusion in Chapter 4. These low-quality diets resulted in low voluntary intake (1.6% DMI/kg LW) and a low animal daily LW gain (DLWG) of 0.18 kg/d (Chapter 3), compared to the experiment reported in Chapter 5 (2% DMI/kg LW and 0.34 kg/d DLWG). It was apparent that on low-quality grass (Chapter 3), an increase in *Desmanthus* inclusion level linearly increased DMI and maintained LW. However, on a high-quality diet as described in Experiment 2 of Chapter 5, increasing the level of *Desmanthus* inclusion did not improve DMI/kg LW beyond 2.2%. When compared to a similar CP diet containing lucerne, a high nutritive value temperate legume, the diets containing *Desmanthus* showed a lower feed intake, which resulted in a lower DLWG of 0.34 kg/d compared to 0.6 kg/d for the animals on lucerne. These findings confirm the reports of Kanani et al. (2006) and Sonawane et al. (2019) that animal performance on *Desmanthus*-containing diets (*D. bicornutus* and *D. virgatus*) was less than on diets containing lucerne or concentrates due to lower digestibility and voluntary consumption of *Desmanthus* diets. However, in both studies, the stage of growth was not provided, making it difficult to make a direct comparison. The reduction in voluntary feed intake can be due to astringent tannins that make *Desmanthus* less palatable (Naumann et al., 2017). Generally, a depressed feed intake is seen at a dietary condensed tannins (CT) concentration exceeding 5% of DM (Naumann et al., 2017), but other previous studies reported lower feed intakes at even lower concentrations of CT (Dschaak et al., 2011; Grainger et al., 2009). Thus, some plant CT seem to be more effective than others at protein binding as palatability is often based on astringency associated with CT-protein complexes formed from salivary proteins (Naumann et al., 2017). In the studies reported in this thesis, the addition of a tannin-binding compound, PEG-4000, did not have an impact on DMI (Chapter 3 and 4).

Crude protein in the diet is correlated to rumen NH_3-N concentration (Brandao and Faciola, 2019; Detmann et al., 2009). Ensuring adequate rumen NH_3-N concentration to supply the majority of N for supporting microbial growth is the first priority in optimising fermentative digestion of forages (Leng, 1990). Satter and Slyter (1974) suggested that the optimal ruminal NH_3-N concentration for maximum microbial growth was 5 mg/dL. Detmann et al. (2014) reported a rumen NH_3-N concentration of 6.3 mg/dL. Rumen NH_3-N concentrations in JCU1 and JCU4 diets increased to 8.0 and 6.9 mg/dL respectively, with an increase in *Desmanthus* proportion in the diet to 31% (Chapter 3), while in the experiments presented in Chapters 4 and 5 on higher quality diets, NH_3-N was higher in animals fed *Desmanthus* (15.8 mg/dL) and lucerne (17.6 mg/dL). The difference in DMI between the lucerne and *Desmanthus* diet might be due to the presence of tannins in *Desmanthus* as the greater the proteins bound by CT, the greater the astringency and the lower palatability (Naumann et al., 2017). Another negative aspect of tannins is its binding properties especially to fibre-degrading enzymes or dietary carbohydrates which decrease rumen turnover rate and negatively impact intake and animal performance (Aboagye and Beauchemin, 2019). For instance, Ahnert et al. (2015) reported a lowered apparent total tract organic matter digestibility at a concentration of Quebracho tannin extract over 4% DM intake in fully grown heifers. They also observed a more pronounced decrease by about 20% for NDF and ADF digestibility at 6% Quebracho tannin extract. Martello et al. (2020) also reported a linearly decrease in *in vitro* DM digestibility and quadratically decreased *in vitro* NDF digestibility with the increasing tannin concentration (0, 0.1 and 0.2% DM tannin) in a protein energy supplements with urea. The lower concentration of rumen NH_3-N in the *Desmanthus* diets compared to the lucerne diet can be due to the binding of free tannins to dietary soluble proteins inducing a decrease in rumen NH_3-N concentration and increase the flow of rumen undegraded protein to the lower tract (Aboagye and Beauchemin, 2019; Bhatta et al., 2009).

Lack of significant differences in CH_4 emissions between *Desmanthus* cultivars was observed between animals supplemented with JCU1 and JCU4 (Chapter 3), and JCU2, JCU4 and JCU7 (Chapter 5). The results disagreed with the *in vitro* findings of Vandermeulen et al. (2018) and Durmic et al. (2017). Vandermeulen et al. (2018) showed higher CH_4 emissions with JCU2 compared to JCU1 and JCU4

after 72 h (Chapter 5). Durmic et al. (2017) reported a similar *in vitro* CH_4 emission with JCU2 and JCU4, but a lower CH_4 emission with JCU1. However, both studies were conducted *in vitro* with a different level of *Desmanthus*. Even with the same tannin diets, previous studies showed discrepancies between *in vitro* and *in vivo* CH_4 emissions. For instance, El-Zaiat et al. (2020) reported a CH_4 production (expressed as mL/kg in vitro DOM) abatement of 29% *in vitro* on a diet containing only berseem (*Trifolium alexandrinum L.*) hay or 50% berseem hay mixed with 50% *Acacia saligna*, *Leucaena leucocephala* or *Atriplex halimus* with rams' rumen fluid on a DM basis. They reported an abatement of only 9% on CH_4 emissions (expressed as L/kg DOM) *in vivo* with the diets containing 50% *Acacia saligna* and *Leucaena leucocephala*. Moreover, they always observed a decrease in CH_4 emissions *in vitro* regardless of the unit used (mL/g DM, mL/kg *in vitro* DOM, mL/kg *in vitro* dry NDF) which was not the case *in vivo* for the CH_4 yield expressed as L/kg DMI where no difference was observed between the control treatment and the diet containing leucaena. The higher CH_4 yield with the *leucaena* diet could be explained by the higher concentration of tannins in the diet which lowered the DMI and increased the CH_4 yield expressed as per kg DMI. Another study conducted by Zhang et al. (2021) evaluating the effect of HT in mitigating ruminant CH_4 *in vitro* and in vivo reported a linear decrease in CH_4 emissions both *in vitro* and *in vivo* with the increasing level of HT in the diet (0, 1.5, 3 and 6% DM). They observed an abatement of 30% *in vitro* expressed as mL/g DM and 36% *in vivo* expressed as L/day or L/day/$W^{0.75}$ with 6% HT in the diet. However, they did not report CH_4 yield expressed as per kg DMI. As feed intake is one of the key factors accounting for variation in CH_4 emissions (Benaouda et al., 2020; van Lingen et al., 2019), it seems that *in vitro* studies constitute good preliminary studies but *in vivo* studies are needed to better understand the impact of the feed on DMI especially in the case of *Desmanthus* which can negatively affect DMI due to its tannin content.

The first CH_4 emission study (Chapter 3) showed a linear decrease in CH_4 yield with increasing level of *Desmanthus* in the diet (CH_4 yield (g/kg DMI), $Y = 19.92 - 0.066\ X$), where X = percentage of *Desmanthus* in the diet. Vandermeulen et al. (2018) attributed the *in vitro* CH_4 abatement with *Desmanthus* supplementation to the presence of plant secondary compounds (CT and/or HT). Previous *in vitro* and *in vivo* studies also found a reduction in CH_4 emissions with tannins or total phenolics

(Aboagye and Beauchemin, 2019; Fagundes et al., 2020a; Hashem et al., 2020). In this thesis, it was demonstrated in Chapters 3 and 5 that TP and CT concentrations were higher in JCU4 compared to the other *Desmanthus* cultivars. Vandermeulen et al. (2018) showed a higher TP concentration in JCU1 than in JCU2 *Desmanthus* cultivars. The results in Chapter 3 showed a negative correlation between CT and CH_4 yield in animals on a low quality diet supplemented with *Desmanthus* (CH_4 yield (g/kg DMI) = 19.67 – 1.49 X, where X = percentage of CT in the diet). Theoretically, the addition of PEG was expected to remove the ability of tannins to inhibit methanogenesis and to bind proteins. However, the results in Chapter 3 showed that the addition of PEG had no impact on CH_4 yield. It is possible that the anti-methanogenic effect observed in that trial was probably due to an improved N availability in the rumen by the inclusion of *Desmanthus* that had a higher digestibility and CP than the basal hay ration. It has been reported that higher quality diets decreased CH_4 emission (Hoffmann et al., 2021) due to a lower fibre content, higher DMI, faster rate of passage from the rumen (Beauchemin et al., 2008) which can be due to the increase in bacterial populations involved in hemicellulose and pectin degradation and ammonia assimilation with the addition of a N-supplement in a poor diet quality (Martinez-Fernandez et al., 2020). Moreover, Blaxter and Clapperton (1965) showed that improvement in diet quality provided by the introduction of legumes in the diet induces an increase in voluntary intake of the animals and promotes more efficient post-ruminal digestion which reduces the energy loss of the diet converted to CH_4.

In **Chapters 4 and 5**, a comparative feeding trial utilising *Desmanthus* and lucerne, the widely characterised and highly nutritive temperate legume, in iso-nitrogenous (similar CP) diets to better understand CH_4 emission patterns, was studied. The results showed no significant difference in CH_4 emissions between the lucerne and *Desmanthus* diets, in contrast to the *in vitro* results of Durmic et al. (2017) which showed a 27% decrease in CH_4 emissions from *Desmanthus* compared to lucerne. The lack of difference in CH_4 emissions between the lucerne and *Desmanthus* diets could potentially be due to a combination of the higher quality and digestibility of lucerne and a low concentration of plant secondary compounds in *Desmanthus*. McCaughey et al. (1999) reported that improving pasture quality using lucerne-grass pastures instead of grass only pastures could reduce CH_4 production by up to 10%

due to a higher digestive efficiency which induces a lesser loss of energy through eructation of CH_4. The low plant secondary compounds concentrations in the diets (lower than 2% for both CT and TP) can also explain the lack of reduction in CH_4 yield in *Desmanthus*. For instance, Beauchemin et al. (2007a) reported no reduction in CH_4 emissions in growing cattle supplemented with 2% quebracho tannin extract regardless of the observed protein-binding effect. The low tannin concentration can also explain the lack of significant difference in CH_4 emissions with the addition of PEG to the diets. However, the addition of PEG increased the rumen concentration of isoacids (Chapter 3 and 5), an indication of increased proteolysis in the rumen through PEG nullifying the rumen protein-binding capacity of tannins. Methane and propionate are usually negatively correlated due to competition for hydrogen (Bhatta et al., 2009). The formation of acetic and butyric acids induces the production of H_2 and CO_2, whereas propionic acid production requires a net uptake of H_2 resulting in a decrease in methanogenesis (Benchaar et al., 2001). The first open-circuit respiration chamber experiment showed the opposite trend with an increase in acetate and a decrease in propionate as the level of *Desmanthus* in the diet increased (Chapter 3). This could be due to the high concentration of NDF as the concentration of rumen acetate increases with the concentration of NDF in the diet which induces a decrease of rumen propionate (Brandao and Faciola, 2019).

Plasma metabolites can provide a suite of predictive biomarkers for livestock health, performance and disease (Connolly et al., 2020; Goldansaz et al., 2017). In Chapter 4, it was shown that NEFA increased in *Desmanthus*-fed cattle (1.66-fold) compared to the lucerne diet, corresponding to a 14% reduction in ME intake. Glucose concentration was similar in all treatments in agreement with the findings of Russel and Wright (1983) and Clemmons et al. (2017) who attributed the lack of significant difference between low and high RFI to the tight regulation of glucose in ruminants and its sensitivity to stress.

Previous studies showed a shift in N loss from urine to faeces due to the aptitude of tannins to complex with proteins and thus decrease the degradability of CP in the digestive tract (Grainger et al., 2009; Lagrange et al., 2020; Tseu et al., 2020). The emission factor for faeces (0.15%) is lower than that of urine (0.26%) as faecal N is mainly in the organic form which is less volatile than urinary N which is subject to nitrification and losses to groundwater (leaching) (Grainger et al., 2009). My study showed a

lesser faecal N in animals fed *Desmanthus spp*. compared to those fed lucerne which would be in favour of a better N utilisation. However, the addition of PEG showed an increasing trend of faecal N. Mkhize et al. (2018) reported contrasting results of a decrease in faecal N in grazing goats supplemented with PEG compared to water or CT. The results from PEG addition should be interpreted carefully because they were obtained in only one period where the diet was of a higher quality as the diet N was higher. This higher dietary N might explain the higher faecal N concentration as N excretion by beef cattle is positively correlated with dietary N intake.

Although no reduction in DMI was observed in Chapter 3, OC can induce a reduction in feed intake such as in Experiment 1 of Chapter 5 and thus CH_4 yield due to animal intrusiveness (Llonch et al., 2016; Llonch et al., 2018), an evaluation of methane emission using the GEM system was conducted in Chapter 5. Minor differences in the average values of CH_4 emissions with the GEM technique compared with OC have been reported (Doreau et al., 2018). GEM also has the advantage of having low labour input and interference with animal behaviour and production (Waghorn et al., 2016). In Chapter 5, the results showed higher CH_4 yields in the 15 and 30% *Desmanthus* treatments compared to the 0% *Desmanthus* (control) diet in contrast to the *in vivo* experiments in Chapter 3 and the *in vitro* experiments by Durmic et al. (2017) and Vandermeulen et al. (2018). The difference in results can be attributed to limitations associated with the GEM evaluation technique. Previous studies showed that it was necessary to take into account different parameters in order to obtain accurate measurements and detect differences in CH_4 emissions (Arbre et al., 2016; Arthur et al., 2017; Denninger et al., 2019; Manafiazar et al., 2017). For instance, Arbre et al. (2016) showed that a minimum of 6-8 animals per group and a measurement period of 17 days were necessary to be able to detect a difference of 20% in CH_4 yield among treatments. Manafiazar et al. (2017) reported that a minimum of 20 samples per animal was required to produce repeatable and reliable CH_4 and CO_2 emission correlations with DMI. Arthur et al. (2017) stated that visits with a minimum of 3-min GEM duration showed fewer variances than summed visits of shorter duration. Regardless of these two factors, the results in Chapter 5 did not change because the 0% *Desmanthus* treatment (Control) remained the one producing the least CH_4. The increase in CH_4 emissions with increasing level of *Desmanthus* in the diet could also be due to the

experimental design as most of the animals in the Control treatment were evaluated by the same GEM compared to the other treatments that were measured by 2 different GEM.

Chapter 7 : Conclusion and Recommendations for Future Research

Together, the findings in this thesis demonstrated that there were no differences in CH_4 emission, feed intake, LW gain, rumen VFA and plasma metabolite profiles between *Desmanthus* cultivars (JCU1, JCU2, JCU4 and JCU7). As a supplement in a low-quality basal diet, *Desmanthus* slightly increased DMI, thus maintaining the LW of the animals and decreasing CH_4 emissions without negatively affecting rumen fermentation and plasma metabolite profiles. In contrast, supplementation of animals on a high-quality diet of Rhodes grass and lucerne with *Desmanthus* did not reduce CH_4 emissions, but improved LW gain. The tannins in *Desmanthus* improved N utilisation due to protein-binding effect, but the ability of these tannins to reduce CH_4 emissions was not evident irrespective of the evaluation technique, particularly with incremental levels of *Desmanthus* inclusion in the diet.

The findings of this thesis will aid Australian beef cattle producers in northern Australia and research scientists when:

- Selecting a tropical legume that can grow in the drier parts of northern Australia (Northern Territory, Queensland and northern New South Wales)
- Reaffirming that *Desmanthus* does not negatively affect animal health or productivity as indicated by rumen VFA and plasma metabolite profiles.
- Demonstrating that *Desmanthus* can maintain DMI, LW gain when offered with a low-quality diet such as what is found in northern Australia.

Future research is recommended for a better understanding of:

(1) The biological role of *Desmanthus*' plant secondary compounds as compounds in mitigating CH_4 emissions and improving feed intake, N retention, rumen VFA and plasma metabolite profiles under grazing systems;

(2) The impact of *Desmanthus*' growth stage on chemical composition;

(3) The effect of supplementing beef cattle with *Desmanthus* on meat quality traits.

In conclusion, the overarching hypothesis that supplementing tropical beef cattle with *Desmanthus* would improve feed intake, animal growth, N utilisation and reduce CH_4 emissions without negatively impacting rumen VFA and plasma metabolites profiles can be partially accepted. It can be accepted if the animals are on a low quality basal diet, but rejected on a high-quality diet such as lucerne.

Limitations of the study:

- The effect of PEG was evaluated in only one experimental period using only two animals in each treatment without enough replication. Future research with longer periods of supplementation with PEG and more animals will help to better understand the role of tannins on CH_4 emissions, animal performance, rumen VFA and plasma metabolites.

- The lack of measurement of the four different levels of *Desmanthus* in the GEM experiment with the same machine was an added unaccountable variable. Thus, data from that study need to be interpreted with caution.

- The lack of reference methods to analyse plant secondary compounds and their sensitivity to external factors such as temperature and light make the plant secondary compounds difficult to reproduce. The measure of biological activity of tannins might constitute a better way to understand how tannins are affecting the animal's CH_4 emissions and performance.

- Fresh field cut and transported *Desmanthus* was used over the study period with some unavoidable variation in growth stages/environment over time. The aim of using fresh material was to simulate grazing of fresh *Desmanthus* however to eliminate variation in forage quality over time perhaps future studies may consider baling *Desmanthus* at a particular stage of growth so as to provide uniform feed base as with the lucerne and Rhodes hay.

- The length of the periods in the experiment of Chapter 3 and the changing in cultivars between periods in the Experiment 1 of Chapter 4 did not allow an accurate capture of weight change.

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Appendices

This section contains all the information on published chapters.

Appendix 1

animals

MDPI

Review

Methane Emissions and the Use of *Desmanthus* in Beef Cattle Production in Northern Australia

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Simple Summary: An in-depth review of Australia's tropical beef cattle production system is presented with emphasis on the use of *Desmanthus*, a tropical legume, as a nutritional supplementation strategy for the abatement and mitigation of methane emissions. It also identifies current knowledge gaps in *in vivo* methane emissions research.

Abstract: The Australian beef industry is a major contributor to the economy with an estimated annual revenue generation of over seven billion dollars. The tropical state of Queensland accounted for 48% of Australian beef and veal production in 2018. As the third biggest beef exporter in the world, Australia supplies 3% of the world's beef exports and its agricultural sector accounts for an estimated 13.2% of its total greenhouse gas emissions. About 71% of total agricultural emissions are in the form of methane and nitrous oxide. In this review, an overview of the carbon footprint of the beef cattle production system in northern Australia is presented, with emphasis on the mitigation of greenhouse gases. The review also focuses on the tropical legume, *Desmanthus*, one of the more promising nutritional supplements for methane abatement and improvement of animal growth performance. Among the review's findings is the need to select environmentally well-adapted and vigorous tropical legumes containing tannins that can persistently survive under the harsh northern Australian conditions for driving animal performance, improving meat quality and reducing methane emissions. The paper argues that the use of appropriate legumes such as *Desmanthus*, is a natural and preferred alternative to the use of chemicals for the abatement of methane emanating from tropical beef cattle production systems. It also highlights current gaps in knowledge and new research opportunities for *in vivo* studies on the impact of *Desmanthus* on methane emissions of supplemented tropical beef cattle.

Keywords: methane emission; tropical beef cattle; *Desmanthus*; supplementation; growth performance; ruminant nutrition; legumes

1. Introduction

Global climate change is principally caused by greenhouse gas (GHG) emissions that result in warming of the atmosphere [1]. According to the Australian National Greenhouse Accounts [2], 13.2% of GHG emissions emanate from agriculture, with methane and nitrous oxide accounting for 71% of

Appendix 2

animals

MDPI

Article

Supplementing Northern Australian Beef Cattle with *Desmanthus* Tropical Legume Reduces In-Vivo Methane Emissions

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Simple Summary: The problem addressed in this study is that of mitigating methane emissions by tropical beef cattle with the aim of reducing the impact of climate change and greenhouse gas emissions in Northern Australia. The primary objective was supplementing tropical beef cattle on poor quality hay with incremental levels of *Desmanthus leptophyllus* cv. JCU1 and *Desmanthus bicornutus* cv. JCU4 to evaluate their in-vivo antimethanogenic effect. Results showed that, irrespective of cultivar, incremental supplementation with up to 31% of *Desmanthus* led to a 10% linear decrease in methane emissions without reducing dry matter intake. This finding makes a significant novel contribution to a better understanding of the impact of supplementing beef cattle with *Desmanthus* on in vivo methane reduction and the role of condensed tannins in rumen fermentation. The practical implication of this finding is that *Desmanthus*, an adapted tropical legume, has the potential to mitigate in vivo methane emissions by beef cattle in the drier parts of Northern Australia and contribute to the larger global effort of reducing the impact of climate change and greenhouse gas emission.

Abstract: The main objective of this study was to investigate the effect of supplementing beef cattle with incremental levels of *Desmanthus leptophyllus* cv. JCU1 and *Desmanthus bicornutus* cv. JCU4 on in vivo methane (CH_4) emissions and the role of tannins in rumen fermentation. Fourteen yearling Droughtmaster steers were allocated to each of the two *Desmanthus* species and offered a basal diet of Rhodes grass (*Chloris gayana*) hay plus fresh *Desmanthus* at 0%, 15%, 22%, and 31% of dry matter intake (DMI). The 15% and 31% *Desmanthus* periods lasted 21 days and the 22 and 0% *Desmanthus* periods, 14 days. Methane production was measured by open-circuit gas exchange in the last two days of each period. The results showed a linear increase in DMI and reduction in CH_4 yield with the increasing level of *Desmanthus* and subsequently condensed tannins in the diet. The added tannin binder polyethylene glycol-4000 did not affect CH_4 yield but increased rumen NH_3-N and iso-acid concentrations. Therefore, on a low-quality diet, *Desmanthus* has the potential to increase intake and reduce CH_4 emissions. Even though its tannins can bind rumen proteins, the beef cattle anti-methanogenic response to supplementation with *Desmanthus* may be a combination of rumen fermentation and tannin effects.

Keywords: methane emission; mitigation; tannins; tropical beef cattle; *Desmanthus leptophyllus*; *Desmanthus bicornutus*; phenolics; legumes; polyethylene glycol; greenhouse gas

Appendix 3

metabolites

MDPI

Article

Plasma Metabolites, Productive Performance and Rumen Volatile Fatty Acid Profiles of Northern Australian *Bos indicus* Steers Supplemented with *Desmanthus* and Lucerne

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Abstract: The hypothesis tested was that tropical steers supplemented with the *Desmanthus* legume and lucerne, a widely characterized temperate legume of high nutritive value, would elicit similar responses in plasma metabolite profiles, productive performance, nitrogen retention, and volatile fatty acids (VFA). The tannin-binding compound, polyethylene glycol-4000 (PEG), was added to the diets (160 g/kg *Desmanthus* dry matter) with the objective of further exploring nitrogen (N) utilization in the animals supplemented with *Desmanthus* relative to lucerne. From February to June 2020, sixteen yearling Brangus steers (average liveweight of 232 ± 6 kg) were fed a background diet of Rhodes grass (*Chloris gayana*) hay for 28 days, before introducing three *Desmanthus* cultivars (*Desmanthus virgatus* cv. JCU2, *D. bicornutus* cv. JCU4, *D. leptophyllus* cv. JCU7) and lucerne (*Medicago sativa*) at 30% dry matter intake (DMI). Relative to the backgrounding period, all supplemented steers exhibited similar growth performance. Steers supplemented with *Desmanthus* recorded a lower DMI and animal growth performance, but higher fecal N concentration than animals supplemented with lucerne. Among the three *Desmanthus* cultivars, there were no significant differences in N concentrations, VFA, and plasma metabolite profiles. The addition of PEG induced higher rumen iso-acid concentrations and fecal N excretion. However, feeding *Desmanthus spp.* to tropical *Bos indicus* steers could be a valuable means of increasing N utilization, which is attributable to the presence of tannins, and, consequently, improve animal productive performance. Since supplementation with lucerne resulted in higher liveweight, daily liveweight gains, and overall animal performance than supplementing with *Desmanthus*, the tested hypothesis that both supplements will elicit similar animal performance does not hold and must be rejected. Further in vivo investigation is needed to better understand the impact of tannins in *Desmanthus* on N utilization.

Keywords: *Desmanthus virgatus*; *Desmanthus leptophyllus*; *Desmanthus bicornutus*; volatile fatty acids; plasma metabolites; legumes; tropical beef cattle; polyethylene glycol; nitrogen metabolism

1. Introduction

"Metabolomics" as a research discipline, is a term derived from "the study of metabolites", which comprehensively measures the end-products (small molecule metabolites) of complex in vivo metabolic processes such as glucose, urea, non-esterified fatty acids (NEFA), bilirubin, aspartate aminotransferase (AST), etc., in cells, biofluids and tissues

Appendix 4

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Response to Climate Change: Evaluation of Methane Emissions in Northern Australian Beef Cattle on a High Quality Diet Supplemented with *Desmanthus* Using Open-Circuit Respiration Chambers and GreenFeed Emission Monitoring Systems

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Simple Summary: The beef industry in Northern Australia is characterized by an extensive grazing system in dry tropical rangelands defined by climate change indices of very low rainfall, a prolonged dry season and feeds of low nutritive value. In response, beef cattle need to be more efficient in converting the available drought-tolerant feeds to muscle, in an attempt to minimize greenhouse gas emissions. This study addressed the problem of reducing methane emissions from tropical beef cattle with the goal of decreasing the impact of climate change and greenhouse gas emissions in Northern Australia. The primary objective was to compare the effect of supplementing tropical beef cattle with both good quality lucerne and poor quality hay with increasing levels of different *Desmanthus* cultivars on in vivo methane emission. The results showed that in tropical beef cattle on high-quality diets, irrespective of cultivar and emission evaluation method, *Desmanthus* does not reduce methane emissions.

Abstract: The main objective of this study was to compare the effect of supplementing beef cattle with *Desmanthus virgatus* cv. JCU2, *D. bicornutus* cv. JCU4, *D. leptophyllus* cv. JCU7 and lucerne on in vivo methane (CH_4) emissions measured by open-circuit respiration chambers (OC) or the GreenFeed emission monitoring (GEM) system. Experiment 1 employed OC and utilized sixteen yearling Brangus steers fed a basal diet of *Rhodes grass* (*Chloris gayana*) hay in four treatments—the three *Desmanthus* cultivars and lucerne (*Medicago sativa*) at 30% dry matter intake (DMI). Polyethylene glycol (PEG) was added to the diets to neutralize tannin binding and explore the effect on CH_4 emissions. Experiment 2 employed GEM and utilized forty-eight animals allocated to four treatments including a basal diet of *Rhodes grass* hay plus the three *Desmanthus* cultivars in equal proportions at 0%, 15%, 30% and 45% DMI. Lucerne was added to equilibrate crude protein content in all treatments. Experiment 1 showed no difference in CH_4 emissions between the *Desmanthus* cultivars, between *Desmanthus* and lucerne or between *Desmanthus* and the basal diet. Experiment 2 showed an increase in CH_4 emissions in the three levels containing *Desmanthus*. It is concluded that on high-quality diets, *Desmanthus* does not reduce CH_4 emissions.