Methane production and productivity changes associated with defaunation in ruminants

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Executive Summary

With increasing world population, global demand for a secure and growing food supply challenges the livestock producers of today to increase output of milk and meat while reducing the environmental impact of animal production. This thesis reports a review of literature and targeted new research assessing the consequences of eliminating rumen protozoa (defaunation) on the performance, digestive function and emissions of the greenhouse gas methane, by livestock.

- Comparative studies of rumen fermentation and animal growth were conducted in growing Merino lambs, crossbred sheep and Brahman cattle. In these studies ruminants were defaunated using coconut oil distillate to suppress protozoa then dosed with sodium 1-(2-sulfonatooxyethoxy) dodecane in a protocol that suppressed feed intake for an average of 10 days but had no detrimental effects on animal health.
- Reflecting the diversity in published literature, these studies found inconsistent effects of defaunation on volatile fatty acid (VFA) concentrations and proportions. Averaged over all experiments conducted, defaunation was associated with a small (5%) reduction in total VFA concentration and an increase (5%) in the ratio of acetate to propionate in the rumen.
- While effects on VFA were not consistent, an average 30% reduction in rumen ammonia concentration and a 16% increase in microbial crude protein outflow (estimated by allantoin excretion) were apparent, suggesting substantial differences in the ruminal degradation and outflow of protein due to defaunation. These changes were associated with an 18% increase in average daily gain (ADG), but surprisingly no increase in wool growth rate.
- Defaunation was associated with a lower enteric methane emission (average 20% reduction) compared to faunated ruminants, with the first studies of daily methane production (DMP) ever made while grazing, made using GreenFeed Emission Monitoring (GEM) units, confirming a 3% lower DMP

(non-significant; $P > 0.05$) and a 9% lower methane yield (MY; CH_4/kg DMI; $P = 0.06$) in defaunated sheep.

- Protozoa affected the rumen response to nitrate, with the nitrate induced reduction in MY being 29% greater in faunated compared to defaunated lambs.
- With dietary coconut oil, no interaction with defaunation was apparent with both coconut oil and defaunation significantly reducing DMP and MY in cattle.
- While defaunation tended to increase average daily gain and reduced enteric methane emissions in cattle by 10%, establishing defaunated cattle proved difficult and is a major constraint to expanding defaunation into commercial herds.
- Assessment of the distribution of protozoa in the forestomaches showed that the number of entodiniomorph protozoa attached to the 'leaves' of the bovine omasum was at least as great as the number attached to the entire surface of the rumen, though all tissue-attached populations are far fewer than the population in the rumen fluid.
- It is concluded that defaunation alone or in combination with dietary supplements of nitrate is effective in decreasing methane emissions, while increasing microbial protein supply and ADG. Commercial implementation of defaunation for cattle will not be able to rely on addition of surfactants to the rumen and it is suggested a bioactive compound distributed through the blood may be needed to remove protozoa residing in the omasum.

In the 6 experimental chapters reporting the research that has been (Chapters 2, 4, 5, 6 and 7) or will be (Chapter 3) submitted for publication, all definitions of abbreviations have been provided anew in each chapter as required for publication. Despite some chapters already been published, minor grammatical and textual improvements have been made (eg. Such as numbering of tables and figs) to improve the clarity and uniformity of these manuscripts when considered within the context of this thesis.

Declaration

I declare that the work presented in this thesis is, to the best of my knowledge and belief, original and my own work, except as acknowledged in the text, that it accurately and truthfully reflects the information gathered during the research program I have undertaken, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

Son Hung Nguyen
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List of abbreviations

ADF	Acid detergent fibre
ADG	Average daily gain
CH_4	Methane
CO	Coconut oil
CO_2	Carbon dioxide
CP	Crude protein
CWG	Clean wool growth
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
DMP	Daily methane production
FCR	Feed conversion ratio
GEM	GreenFeed emission monitor
GHG	Greenhouse gases
H_2	Hydrogen
LC	Lucerne chaff
LW	Liveweight
MCFA	Medium-chain fatty acid
ME	Metabolisable energy
MetHb	Methaemoglobin
MI	Methane intensity
MJ	Mega joules
MP	Methane production
MY	Methane yield
N	Nitrogen
NDF	Neutral detergent fibre
NO_3	Nitrate
NPN	Non protein nitrogen
OC	Oaten chaff
OM	Organic matter
RR	Reticulo-rumen
VFA	Volatile fatty acid

Chapter 1

Review of the literature

1.1 Overview

Australia's agricultural sector produces an estimated 87.4 Mt of greenhouse gas emissions (GHG; expressed as CO_2 equivalent; CO_2-e) annually, contributing 16% of national GHG emissions. Enteric fermentation is the main source of agricultural GHG emissions accounting for 64.3% of Australia's agricultural emissions (56.2 Mt CO_2-e; Figure 1.1). Livestock enteric fermentation is not only Australia's largest agricultural GHG emission source but it also contributes 11.6% of GHG from anthropogenic sources globally (Ripple *et al.* 2014). In association with an increased global human demand for food, agricultural demand for livestock products and consequently GHG emissions are expected to increase in coming years (van Beek *et al.* 2010). Therefore, mitigation strategies should focus on increasing animal efficiency and decreasing GHG emissions per unit of edible food (Pinares-Patiño *et al.* 2009). Reduction of methane emissions per unit of animal product will be achieved by means of reducing the proportion of energy consumed by livestock that is expended in maintenance and directing more towards faster growth, milk yield and shorter dry period in lactating cows (Monteny *et al.* 2006).

Figure 1.1 Greenhouse gas sources from Australia's agricultural sector (Thousand tonnes CO_2 equivalents; Department of the Environment 2014).

1.1.1 The making of enteric methane

The chemical pathways of methane (CH_4) formation in the rumen are demonstrated in Figure 1.2. In summary, nicotinamide adenine dinucleotide (NADH) is produced by glycolysis in ruminal microbes during fermentation, associated with pyruvate production. Pyruvate is further metabolised by ruminal microorganisms to produce volatile fatty acid (VFA), mainly acetate, propionate and butyrate. Formation of acetate and butyrate occur through pyruvate-formate lyase which produces formate and acetyl CoA. Acetyl CoA is metabolised by rumen microbes to produce acetate and butyrate while the formate is catabolised to CO_2 and hydrogen (H_2) which are also used to

produce CH_4 (Leng 2014). Formation of propionate occurs via pyruvate which is converted to propionate by either the oxaloacetate or lactate pathway (Figure 1.2). During fermentation, NADH is reoxidised NAD^+ with the production of H_2 [NADH + $H^+ \leftrightarrow NAD^+ + 2(H)$] so that glycolysis and fermentation continues (Nolan 1999). Rumen methanogens cannot use propionic or butyric acid for their energy substrates, but H_2, CO_2, formic acid, acetic acid and some methanol can be used by methanogenic archaea for their energy requirement leading to CH_4 excretion. Acetic acid is converted to CO_2 and CH_4 by the methanogens with cytochromes while H_2, CO_2 and formate are converted to CH_4 by the methanogens lacking cytochromes (Thauer *et al.* 2010).

Figure 1.2 Pathways leading to VFA and methane production ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$) in glucose (Nolan 1999).

1.1.2 The management of enteric methane

Enteric CH_4 is a by-product of microbial fermentative digestion in the rumen of ruminants that also represents a loss of 5 to 7% of gross energy intake, equivalent to a CH_4 yield of 16 to 26 g CH_4/kg of dry matter (DM) consumed (Hristov *et al.* 2013). In addition, animals, that grow faster, produce less CH_4 per kg of ADG on the same quality of diets (Figure 1.3). Theoretically, enteric CH_4 emissions from ruminants can be managed or mitigated by several potential strategies as summarised below.

- Diet manipulation to reduce CH_4 release per unit of animal production (Hristov *et al.* 2013). This strategy is achievable by feeding animals highly digestible diets, but it is less feasible in the many developing countries where residues of crops and agricultural by-products are the major feed sources for livestock (Preston 1995).
- Addition of feed additives that inhibit methanogenesis including bromochloromethane (Hristov *et al.* 2013) and plant secondary compounds such as saponins and tannins (Patra and Saxena 2009) or additives such as dietary fatty acids that have a high affinity for bioreduction (Patra 2014). However, these secondary compounds need to be well understood in regard to their sources, absorption, metabolism and biological effects on livestock health and production before they can be used in commercial livestock production systems (Durmic and Blache 2012).
- Removal of the rumen protozoa (defaunation). Methanogens, which exist as endo- and ecto-symbionts with ciliate protozoa (Finlay *et al.* 1994; Tokura *et al.* 1997), had been estimated to account for 37% of CH_4 production (Finlay *et al.* 1994).

Elimination of rumen protozoa can reduce CH_4 emissions (Hegarty 1999; Newbold *et al.* 2015) and increase growth rate and liveweight gain of ruminants (Eugène *et al.* 2004a; Newbold *et al.* 2015) especially when feed is deficient in protein rather than energy content.

- Supplementation of a diet with nitrate as non-protein nitrogen (NPN) source that promotes the growth of bacteria (Lin *et al.* 2011) and has higher affinity for H_2 than does methanogenesis (Leng and Preston 2010).
- Creating an environment in the rumen that encourages the growth of reductive acetogenic (Fonty *et al.* 2007; Ungerfeld 2013) and methanotrophic microbes (Leng *et al.* 2012).

Figure 1.3 The relationship between enteric methane production and liveweight gain of cattle (Kurihara *et al.* 1999).

1.2 The rumen protozoa and classification

Protozoa were first discovered by Leeuwenhoek in 1675, and various protozoa were identified as ciliates as their locomotion was made by the means of small hairs or cilia over the surface of the body. Through evolution, as ruminants consumed grass and water, the protozoa established in stomachs of ruminants and adapted to utilise this habitat (Hungate 1966). Anaerobic rumen ciliates are extremely abundant, ranging from 10^5 to 10^6 cells/mL of rumen liquor and are capable of engulfing bacteria and digesting plant materials such as cellulose and other structural carbohydrates (Finlay and Esteban 2013; Esteban *et al.* 2014).

Rumen protozoa are classified based on their cell morphology, and considered to be the simplest form of animal life, performing all the life processes as a eukaryotic cell (Dehority 2003; Esteban *et al.* 2014). Further, the majority of protozoa identified in the rumen are ciliate species, with more than 100 species of rumen ciliate protozoa having been identified in two major sub-classes; being the entodiniomorphid (Table 1.1) and the holotrich ciliates (Table 1.2; Williams and Coleman 1988, 1992). A few species of flagellate protozoa (Table 1.3) are also found in the rumen (Hungate 1966; Williams and Coleman 1992). However, the flagellates are easily confused with fungal zoospores (Dehority 2003). In addition, flagellate protozoa are less numerous in terms of population density and have small body mass compared to the ciliates (Hungate 1966; Clarke 1977). Therefore, the flagellates are not well known and have not been the focus of attention in classifying or describing their activity and metabolism, leading to little information being available.

Table 1.1 Characteristics of some rumen entodiniomorphid protozoa (Williams and Coleman 1988).

Genus	Dorsal cilia	Obvious skeletal plates	Macronucleus shape	Length (µm)	Width (µm)
Entodinium	0	0	Various	22-29	11-68
Eodinium	1 band ant.end‡	0	Rod-shaped	32-60	20-40
Diplodinium	1 band ant.end	0	Often bent rod	55-210	41-136
Eremoplastron	1 band ant.end	1 narrow	Often bent rod	45-500	21-260
Eudiplodinium	1 band ant.end	1 narrow	Hook shaped	105-198	56-120
Ostracodium	1 band ant.end	1 wide	Various	58-133	36-54
Polyplastron	1 band ant.end	2 narrow	Rod-shaped	123-205	98-123
Diploplastron	1 band ant.end	2 narrow close at post. End	Rod-shaped	88-120	47-65
Metadinium	1 band ant.end	2 narrow occ. Fused	Rod-shaped 2-3 lobes	110-288	61-165
Epidinium	1 band behind ant.end	3 variable width	Elongate	105-150	44-72
Enoploplastron	1 band ant.end	3 narrow close together	Elongate	60-140	32-90
Ophryoscolex	1 band round 3/4 of middle	3 variable width	Elongate	120-215	60-80
Epiplastron	1 band round 3/4 of middle	5 variables width	Elongate	90-140	41-60
Elytroplastron	1 band ant.end	3 narrow (2 on right 1 on left)	Elongate	110-160	67-97
Caloscolex	1 band round all middle	1 complex	Elongate	130-160	73-90
Opisthotrichum	1 band round 1/3 of middle	1 cylindrical	Elongate	60-80	21-28
Parentodium	0	0	Round	26-39	14-21

‡ *anterior end*

Table 1.2 Characteristics of some rumen holotrich ciliates (Williams and Coleman 1992).

Species	Morphology	Size range, average (μm)	Length: width range, average	Macronucleus
Isotricha prostoma	Elongated ovoid to elipsoidal	80-200×50-120; 135×70	1.69-2.55; 2.03	Elongated
Isotricha intestinalis	Elongated ovoid to elipsoidal	90-200×45-150; 110×60	1.65-1.93; 1.76	Ovoid, 30×20
Dasytricha ruminantium	Ovoid	35-75×20-40; 57×27	1.70-2.70; 2.11	Elongated/ellipsoidal, 16-18×8-9
Dasytricha hukuokaensis	Ovoid	120-180×68-122; 151×95	1.47-1.76; 1.59	Ellipsoildal, 24-38×16-20; 31×18
Oligoisotricha bubali	Ovoid	12-20×8-15; 16×12	1.07-1.60; 1.30	Spherical-elliptical
Buetschlia parva	Ovoid	30-67×20-48; 55×35	1.58-2.38; 1.91	Spherical
Buetschlia neglecta	Ovoid	40-60×20-30	2.0	Spherical
Buetschlia lanceolate	Spear-shaded	48×20	2.4	Large
Buetschlia omnivore	Ovoid/spherical	Variable; 35-110×27-97		Elongated
Buetschlia nana	Ovoid	17-21×12-17;19×15		spherical
Parabundleia ruminantium	Ovoid	37.5-50 × 27.5-32.5; 42.5 × 30.5	1.25-1.54	Elliptical, 16 μm long
Polymorphella bovis	Ovoid to bottle-like	26-37.5 × 20-26; 34× 22	1.30-1.80 1.56	Subspherical, 2.5 μm long
Blepharoprosthium parvum	Pyriform	26-32 × 16-20; 29 × 18		Spherical
Blepharoconus krugerensis	Ovoid with anterior knoblike protuberence	30-65 × 21-60; 46 × 35	1.11-1.80; 1.34	Disc-shaped 7-15 × 4-8; 11 × 5.5 μm
Microcetus lappus	Ovoid/elongate	18-29 × 7.5-18; 23.6 × 13		Spherical

Table 1.3 Characteristics of some rumen flagellate protozoa (Williams and Coleman 1992).

Species	Shape	Size	Number of flagella	Size of nuclear (μm)
Chilomastix caprae	Piriform	8.3×4.4	4	
Monocercomonas ruminantium	Piriform	4.8×4.1	4	1.8×1.6
Monocercomonoide bovis	Elliptical	5.4×2.8	4	1.6×1.4
Monocercomonoide caprae	Elliptical	9×6	4	Large
Pentatrichomonas hominis	Elliptical	7.5×5.6	5	2.5×2.0
Tetratrichomonas buttreyi	Elliptical	5.3×4.8	4	2.0×1.7
Trichomonas ruminantium	Elliptical	12×10	3	

1.3 Role of protozoa in ruminant nutrition

Rumen protozoa account for as much as half the total microbial biomass in the rumen and up to 60% of total fermentation products (Williams and Coleman 1992; Newbold *et al.* 2015). They actively participate in the ruminant digestion process. Removing protozoa from the rumen, therefore, may result in modifying ruminal digestion of plant cell walls and starch which are considered to be two main sources of energy supply for ruminants (Jouany and Martin 1997). The ruminal ecosystem and environment can be slightly altered by the absence of protozoa with significant influence on bacterial activity, affecting the retention time of the digesta, the concentrations and proportion of ruminal VFA and ammonia (NH_3) concentration (Eugène *et al.* 2004a; Newbold *et al.* 2015) and therefore the supply of metabolites to the host, especially amino acids.

1.3.1 Plant cell wall digestion

Early studies on the role of rumen protozoa in ruminal digestion concluded that rumen protozoa did not digest plant cell components (Becker 1929). In later years, enzymatic and microscopic evidence showed that cellulose was digested by entodiniomorphs (Hungate 1966). Further, the rumen ciliate protozoa were confirmed in their ability to colonize and damage plant tissues in studies with scanning electron microscopic technology. *Epidinium crawley* was found to cause primary degradation of plant tissues (Bauchop and Clarke 1976) as it attached and damaged areas of the stem. *Entodinium spp*. and *Ophryoscolex caudatum*, however, rarely attached themselves to the large tissue fragments, but to damaged tissues exposed through fractures (Orpin 1984).

Rumen protozoa produce fibre-degrading enzymes and the cellulolytic enzymes produced by rumen protozoa are distinguished from those of bacterial and fungal origin. This was observed by the characterization of protozoal genes encoding cellulase enzymes (Jouany and Martin 1997). Further, polysaccharidase activities were greater in animals with ciliate protozoa compared to animals without ciliate protozoa (Santra and Karim 2002; Eugène *et al.* 2004b). However, effects of rumen protozoa on ruminal digestion are inconsistent in the literature. There are reports of decreased organic matter (OM) digestibility (Eugène *et al.* 2004a; Newbold *et al.* 2015), and neutral-detergent fibre (NDF) and acid-detergent fibre (ADF) digestibility (Newbold *et al.* 2015) in the absence of rumen protozoa, probably due to the loss of protozoal fibrolytic activity. The absence of rumen protozoa, in contrast, increases cellulolytic ruminococci populations (Mosoni *et al.* 2011). This increased cellulolytic ruminococci in the absence of rumen

protozoa may compensate for the loss of protozoal fibrolytic activity, and therefore the digestibility of OM, NDF and ADF was not different between defaunated and faunated animals (Jouany *et al.* 1995). Zeitz *et al.* (2012) observed no effects of individual ciliate protozoa such as *Entodinium caudatum*, *Epidinium ecaudatum* and *Endiplodium maggii* on whole tract digestibility of OM, NDF or ADF and suggested that ciliate protozoa may not always improve plant cell wall digestion.

1.3.2 Carbohydrate and starch digestion

Both holotrich and entodiniomorph ciliate protozoa are believed to ferment carbohydrates to meet their energy requirement. Holotrich ciliates utilise soluble carbohydrates, while *Entodinium spp*. and *Epidinium spp.* preferably digest starch (Williams 1989). De Smet *et al.* (1992) showed that the total protozoal population density was nearly double in a high concentrate diet compared to a high roughage diet, with holotrich ciliates increasing accordingly. *Entodinium spp*. and *Epidinium spp*. have their largest numbers with a high concentrate diet.

Wereszka and Michałowski (2012) found that *Diploplastron affine* possessed enzymes degrading starch and its derivatives. A protozoal cell extract for enzymatic studies found *Diploplastron affine* ciliate capable of digesting starch, released about 45 pmol VFA per protozoa per hour and utilised liberated energy for their energy requirement. The rate of starch degraded by *Diploplastron affine* is equivalent to 2.4 ± 0.47μmol/L glucose per mg protein per min and the degradation rate of maltose is approximately 0.05μmol/L glucose per mg protein per min (Wereszka and Michałowski 2012). The ciliate *Diploplastron affine* is also found to digest insoluble 1,3-ß-glucans such as

pachyman and 1,6-ß-glucans such as pustulan as energy substrates (Belzecki *et al.* 2012).

Apart from being able to digest and utilise plant carbohydrates, rumen protozoa also ferment chitin of the rumen fungus. Morgavi *et al.* (1994) found *Piromyces spp*. strain OTS1 in monocultures or in the presence of rumen protozoa *in vitro* and reported that rumen protozoa adversely affect the growth of *Piromyces spp*. strain OTS1 and are able to digest fungal cell walls, resulting in 42% reduction of chitin which is a carbohydrate component of the fungal cell wall. *Diploplastron affine* and *Entodinium caudatum* are found to possess a chitinolytic enzyme (Miltko *et al.* 2015b) and utilise chitin as a source of energy for ciliate metabolism (Miltko *et al.* 2015a).

1.3.3 Protein digestion and protozoal synthesis in the rumen

Ruminants with protozoa in the rumen (faunated) support a higher ruminal NH_3 concentration than do animals with rumen protozoa removed (defaunated), indicating that rumen protozoa degrade dietary proteins (Jouany 1996) and engulf bacteria for their amino acid requirement (Coleman 1989; Esteban *et al.* 2014). Ueda *et al.* (1975) who incubated the isotrich ciliates with soluble casein found that peptide-nitrogen and amino-nitrogen produced by the isotrich reached its highest level between 3 and 15 hours of incubation, accounting for 47% and 58% of non-protein nitrogen (NPN), respectively. Incubation of the ophryoscolecid ciliates with insoluble casein showed a peak of peptide-nitrogen at 3 hours, which accounted for 36% of NPN while amino-nitrogen increased linearly and accounted for 47% of NPN at 15 hours of incubation (Ueda *et al.* 1975).

Protozoal nitrogen (N) can account for 53% of the total microbial N in the bovine rumen (Michałowski 1979), which is about 24 to 46 g N (Leng *et al.* 1981). However, rumen protozoa contribute only 20% of the total microbial N entering the duodenum (Jouany *et al.* 1988). The smaller protozoal biomass in the duodenum of the ruminants could be due to 65% to 74% of protozoa lysing and being degraded in the rumen (Leng 1982; Ffoulkes and Leng 1988), suggesting only 24 to 35% of protozoa enter the lower digestive tract. The relatively high numbers of rumen protozoa that complete their life span in the rumen (Leng 1982) and are retained within the omasum of ruminants (Czerkawski 1987) mean rumen protozoa contribute a small proportion of the total microbial protein supply. The principal detrimental effect of rumen protozoa, therefore, may be competition for substrate with bacteria and engulfment and digestion of bacteria by protozoa, leading to decreased bacterial biomass and flow of protein in the duodenum (Leng 1982).

1.3.4 Ruminal lipid metabolism

The role of rumen protozoa in bio-hydrogenation is not well defined and understood (Williams and Coleman 1992), although they contribute significantly to flow of unsaturated fatty acids to the duodenum (Newbold *et al*. 2015; Yáñez-Ruiz *et al*. 2006). Yáñez-Ruiz *et al*. (2006) reported rumen protozoa accounted for between 30-40% of conjugated linoleic acid (CLA) and 40% of vaccenic acid (VA) leaving the rumen. Mixed protozoa from the sheep rumen contain at least two to three times more unsaturated fatty acids, including CLA and VA, than do bacteria. Different species have different composition, with larger fibrolytic species such as *Epidinium ecaudatum*

caudatum containing more than ten times more CLA and VA than some small species, including *Entodinium nanellum* (Devillard *et al*. 2009). This high level of polyunsaturated fatty acids in protozoal cells is a consequence of ingestion and/or engulfment of chloroplasts (Huws *et al*. 2009) and this chloroplasts uptake is specifically found in entodiniomorphids (Huws *et al*. 2012). Rumen protozoa, therefore appear to increase the duodenal flow of mono or polyunsaturated fatty acids by protecting chloroplasts unsaturated fatty acids from rumen bio-hydrogenation.

1.4 Factors affecting protozoal population densities in the rumen

Rumen ciliate protozoa represent approximately 10^4-10^6 cells/mL of rumen contents (Dehority 2003; Esteban *et al.* 2014), but the concentration of rumen protozoa varies among animals and is dependent on many factors such as ruminant species, geographical location (Akbar *et al.* 2009), diet (Whitelaw *et al.* 1984), frequency of feeding and rumen pH (Clarke 1977).

1.4.1 Diet composition

Ruminants fed highly digestible diets often show the largest populations of rumen protozoa (Hungate 1966), while small populations of rumen protozoa are found in animals on low quality roughage diets (Abe *et al.* 1973). De Smet *et al.* (1992) fed sheep low and high concentrate diets containing 4.3% or 17.3% starch respectively, observing total protozoal population was nearly two-fold higher in the high concentrate diet. Rumen protozoa are able to reduce the rate of fermentation, contributing to the maintenance of a stable ruminal ecosystem when a high concentration of grain is

suddenly introduced in the diet (Mackie *et al.* 1978). However, the protozoa are significantly affected by the environment's acidity or alkalinity, with the protozoa unable to survive if rumen pH is above 7.8 or below 5.0 (Clarke 1977). Mackie *et al.* (1978) also found that the protozoal population decreased by 50-80% as rumen pH fell below 5.4. Cellulolytic ciliates almost disappeared when cattle were fed barley only (Kudo *et al.* 1990) and steers became protozoa-free for a period of a few weeks by *ad libitum* feeding of barley (Whitelaw *et al.* 1984).

1.4.2 Dietary fatty acid supplement

Capric acid (C10:0), lauric acid (C12:0) and myristic acid (C14:0) show strong protozoal toxicity and are useful rumen defaunating agents (Matsumoto *et al.* 1991). Matsumoto *et al.* (1991) observed that rumen protozoa, except *Entodinium spp*. were undetectable after 3 days of feeding 30 g of hydrated coconut oil (CO) containing 52% lauric acid. Feeding 250g of refined CO to beef heifers reduced rumen protozoal population by 62% (Jordan *et al.* 2006) and protozoal populations in beef heifers were decreased by 63% and 80% by 300 g/d CO after 45 and 75 days, respectively (Lovett *et al.* 2003). Machmüller (2006) reported a reduction in rumen protozoa by 88 and 97% when feeding sheep with 3.5 and 7% CO, respectively. This suppressive effect of CO on rumen protozoa persisted 5 weeks after finishing feeding sheep with CO (Sutton *et al.* 1983). Rumen protozoa were reduced to half of the original population by cottonseed, with holotrich and cellulolytic protozoa apparently lost from the rumen of sheep and only *Entodinium spp*. remained (Dayani *et al.* 2007).

1.4.3 Frequency of feeding

The concentration of protozoa in the rumen liquor varies according to the daily feeding regime, reaching a maximum before feeding and decreasing by approximately 60-80% from 4 to 12 hours after feeding (Michalowski and Muszyński 1978). More specifically, the holotrich population in the fluid decreases for a period of 12 to 20 hours after feeding and the population returns to its original numbers within 4 to 6 hours pre-feeding, while the entodiniomorphid population in the fluid decreases for up to 16 hours after feeding and then increases to the pre-feeding numbers (Williams 1986). The increase of the holotrich population is mainly caused by the increase in dasytrichs while the isotrich population remains relatively low (Clarke 1965). The highest concentration of rumen protozoa occurs when the animal is fed three or four meals per day rather than once (Bonhomme 1990).

1.5 Defaunating the rumen

Rumen protozoa are important, but not essential in the rumen ecosystem and to the well-being of host animals (Williams and Coleman 1992; Newbold *et al.* 2015). Elimination of rumen protozoa (defaunation) has led to reported increases in growth rate and liveweight gain of ruminants (Bird and Leng 1978; Bird *et al.* 1979; Eugène *et al.* 2004a; Newbold *et al.* 2015) especially when the feed is deficient in protein relative to energy content. In addition, rumen protozoa are significant hydrogen (H_2) producers and synthesise mainly acetate and butyrate rather than propionate (Williams and Coleman 1992). Defaunation is therefore expected to induce a greater proportion of propionate in the ruminal VFA (Eugène *et al.* 2004a), but this phenomenon is not always observed

(Williams and Coleman 1992; Newbold *et al.* 2015). The reduced CH_4 emissions caused by defaunation also reported by several authors (Whitelaw *et al.* 1984; Hegarty 1999; McAllister and Newbold 2008; Newbold *et al.* 2015) may reflect reduced H_2 availability by removing endosymbiotic methanogens (Finlay *et al.* 1994; Tokura *et al.* 1997; Finlay and Esteban 2013). To attain desired fermentation and productivity advantages from defaunation, many defaunation strategies have been tried and are summarised below.

1.5.1 Isolation of young ruminants after birth

Rumen ciliates are not observed in new born animals, but that rumen ciliates are passed from mother to baby by direct transfer of saliva containing the active protozoa (Stewart *et al.* 1988). Fonty *et al.* (1986) found protozoa appeared in lambs in the following order: *Entodinium* (15-20 days), *Polyplastron*, *Eudiplodinium* (20-25 days) and *Isotricha* (50 days).

Therefore, rumen ciliate protozoa are not present in animals at birth, enabling protozoa-free animals to be established by separating offspring from their mothers (Ivan *et al*. 1986). Bryant and Small (1960) reared calves isolated from birth which did not have ciliate protozoa until they were inoculated with rumen contents at 24 weeks of age. Eadie and Gill (1971) separated lambs from their dams at 2 days of age and maintained protozoa-free lambs for 61 weeks during the length of the experiment. Dehority (1978) also isolated lambs for almost a year without protozoa until the sheep were inoculated with rumen contents to faunate them. In addition, Hegarty *et al*. (2008) established a flock of ciliate-free lambs born from defaunated ewes and the lambs remained protozoa-

free for an extended period of time while grazing. It seems probable young ruminants can be reared free of protozoa when isolated after birth, but it is time consuming and it is not applicable to adult ruminants.

1.5.2 Chemical drenching methods

Chemicals administered into the rumen by using an oesophageal tube or through a rumen fistula have been shown to eliminate the rumen protozoa. A chemical dosing method was described by Becker (1929) who fasted goats for three days and at the end of 72 hours, goats were dosed with 50 mL of 2% copper sulphate for two consecutive days. Jouany *et al.* (1988) repeated this method and reported 50% of treated sheep died of copper poisoning and only one sheep was protozoa-free for 93 days. This protocol was therefore considered dangerous and unreliable.

Rumen ciliate protozoa are susceptible to surface-active agents and these agents provide an effective protocol for defaunation of the rumen. In sheep, defaunation has been successful with sodium 1-(2-sulfonatooxyethoxy) dodecane (Bird *et al.* 2008; Hegarty *et al.* 2008) or sodium lauryl sulfate (Santra *et al.* 2007a). In cattle, removing protozoa with chemical treatment appears more challenging. Diocyl sodium sulfosuccinate (Manoxol OT) used by Abou Akkada *et al.* (1968), and non-ionic surfactants such as nonyl-phenol ethoxylate (Teric GN9) used by Bird and Leng (1978) were not successful in rendering cattle free of ciliate protozoa for prolonged periods. These surface-active agents are more effective to use in sheep than in cattle, but even defaunating sheep with surfactants is not always successful (Machmüller *et al.* 2003).

Part of the difficulty in chemical defaunation may be due to protozoa residing in the omasum. The omasum transfers digesta from reticulum into the abomasum and has a major role in water reabsorption (Van Soest 1994). The flow of digesta from the reticulum occurs following the omasal canal contractions, but occasionally backflow of large volumes of digesta from the omasum to the reticulum occurs when the omasal body contracts during the closure of omaso-abomasal orifice (Stevens *et al.* 1960). Therefore, Towne and Nagaraja (1990) claimed that the backflow of omasal contents containing residual omasal protozoa would re-inoculate the rumen of the defaunated rumen in steers. Towne and Nagaraja (1990) also suggested that anatomical differences between bovine and ovine omasums affect the efficacy of chemical defaunation because the number of omasal laminae in cattle are almost double those in sheep. Chemicals are therefore less likely to reach all protozoa residing between laminae in bovines than in sheep, and this may explain why reports of successful defaunation in cattle are rare.

1.5.3 Effect of defaunation on extent of ruminal fermentation

An *in vitro* study by Yoder *et al.* (1966) reported cellulose digestion by rumen protozoa (7%), by bacteria (40%) and by protozoa and bacteria combined (exceeded 60%), showing a beneficial effect of rumen protozoa on cellulose digestion. Bauchop and Clarke (1976) observed rumen ciliate protozoa contribute to fibre digestion as they are capable of colonizing and damaging plant tissues. Cellulolytic, polysaccharide depolymerases and glycoside hydrolase enzymes produced by protozoa are significant contributors to cellulose and hemicellulose fermentation (Coleman 1989). Therefore,

the absence of rumen protozoa can lead to a 5-15% reduction in carbohydrate digestion of plant cell walls (Jouany *et al.* 1988).

Removing protozoa reduces the rumen digestibility of fibre components of the diet (Newbold *et al.* 2015). Ruminal digestion of NDF and ADF were reduced by 31% and 22% respectively by defaunation of sheep when fed a low soluble N diet (Ushida and Jouany 1990). Defaunation also reduced degradation of a mainly chopped hay diet by up to 18% *in sacco* (De Smet *et al.* 1992). The absence of rumen protozoa did not affect rumen digestibility in lambs offered a diet with a high protein/energy ratio, but reduced total tract digestibility of OM (10%) and NDF (7%; Eugène *et al.* 2010). In addition, Ushida and Jouany (1990) found that defaunated ruminants require an increased supply of NPN in order to maintain fibrolytic activity in the rumen compared to faunated animals. Although fibre digestion is moderately suppressed by defaunation, improving protein supply is far more important in growing animals with high protein demand and when protein is a limiting factor in the diet (De Smet *et al.* 1992).

1.5.4 Effect of defaunation on ruminal volatile fatty acids

The effects of defaunation on ruminal VFA concentration and the molar proportions of VFA are not entirely consistent within the literature (Williams and Coleman 1992; Newbold *et al.* 2015). Defaunation has been reported to increase total VFA concentration in defaunated sheep (Santra *et al.* 2007a) and weaner lambs (Santra and Karim 2002), but Hegarty *et al.* (2008) reported that animals with protozoa had higher concentrations of total VFA compared with defaunated animals. Molar proportions of VFA are also inconsistently affected by defaunation, although butyrate and acetate

proportions were generally increased (Machmüller *et al.* 2003; Bird *et al.* 2008) and proportion of propionate generally decreased after defaunation (Machmüller *et al.* 2003; Hegarty *et al.* 2008). A higher proportion of acetate and lower proportion of propionate in the VFA of defaunated animals was reported when animals were fed low-quality diets (Bird 1982). In addition, Newbold *et al.* (2015) reported in their meta-analysis that defaunation significantly decreased butyrate and increased acetate, but did not affect propionate. These inconsistent effects of defaunation on VFA concentration and molar proportions may reflect variable effects of defaunation on the bacterial population within the rumen. Defaunation increases the number of bacteria, which induces changes in digestion and fermentation due to bacterial species distribution (Mosoni *et al.* 2011; Zeitz *et al.* 2012) and bacterial composition changes (Ozutsumi *et al.* 2005).

Elimination of ciliate protozoa from the rumen may allow a proliferation of rumen bacteria, leading to increased uptake of NH_3 by bacteria for protein synthesis with less protein degraded by rumen protozoa (Williams and Coleman 1992; Jouany and Ushida 1999; Santra *et al.* 2007a). Decreases in NH_3 concentration in defaunated animals compared to faunated animals were observed in many studies (Eugène *et al.* 2004a; Newbold *et al.* 2015). Less ruminal catabolism of engulfed feed-protein and bacteria occurs in the absence of rumen protozoa, leading to an increase in the supply of protein to the duodenum of ruminants for productive purposes (Bird and Leng 1978; Jouany 1996; Newbold *et al.* 2015).

1.5.5 Effect of defaunation on animal performance

A positive effect of defaunation on ruminants is the increased rumen bacterial biomass and passage of ruminal undegraded protein from the diet (Jouany 1996). Duodenal N and duodenal CP/kg DMI outflow significantly increased after defaunation (Eugène *et al.* 2004a; Newbold *et al.* 2015), indicating an increase in the efficiency of microbial protein synthesis and leading to an average increased daily gain of 11%.

In pen-feeding studies, defaunated lambs showed 18% faster growth rate and greater wool growth and wool fibre diameter over faunated or refaunated lambs offered a 50:50 concentrate and roughage ration (Santra *et al.* 2007b). Birth weight of lambs born from defaunated ewes was 13% heavier than from faunated ewes on single-born lamb and pre-weaning growth rates were 10% and 14% heavier in lambs reared free of ciliate protozoa for both single and twin-born lambs, respectively (Hegarty *et al.* 2008). On high energy and low protein diets, defaunated cattle grew at a 43% faster rate than faunated cattle on the same intake (Bird and Leng 1978) and lambs without rumen protozoa showed significantly increased growth rates and efficiency of utilisation of feed when fed a low level of protein. Wool growth increased by 50% compared to faunated animals that were fed a low protein diet (Bird *et al.* 1979).

In grazing studies, Bird and Leng (1984) observed a greater rate of body weight gain (23%) and wool growth (19%) in defaunated compared to faunated lambs grazed on a green oats pasture. Protozoa-free lambs born from defaunated ewes were significantly (4-8%) heavier than were lambs born from faunated ewes measured from 2 months of

age to 5 months of age and wool growth was also greater in protozoa-free lambs grazed on fescue dominant pastures (Hegarty *et al.* 2000).

However, defaunation has been associated with a 30% increase in rumen volume (Orpin and Letcher 1984) and the increased weight of ruminal contents after defaunation was probably due to longer particle retention of ruminal digesta associated with the rumen fill effect of lower OM digestibility (Eugène *et al*. 2004a). There is little data on carcass and gut weights of defaunated over faunated animals reported in the literature.

1.5.6 Effect of defaunation on enteric methane production

As stated earlier, ciliate protozoa are significant producers of H_2 and produce acetic and butyric acids rather than propionic acid (Williams and Coleman 1992). Defaunation is generally associated with fermentation shifting to a greater proportion of propionic acid, therefore reducing the amount of CH_4 produced (Eugène *et al.* 2004a).

The methanogens existing as endo- and ecto-symbionts with ciliate protozoa (Finlay *et al.* 1994; Tokura *et al.* 1997; Finlay and Esteban 2013) have been estimated to account for 37% of ruminal methane production (Finlay *et al*. 1994). The proportion of methanogens in the total bacterial population was lower in protozoa-free lambs, with 26% lower CH_4 emissions compared to faunated lambs (McAllister and Newbold 2008). While the archaeal community of methanogens in liquid and solid rumen contents were similar in faunated wethers, a lower proportion of methanogens occurred in the liquid phase with defaunation (Morgavi *et al*. 2012). However, Mosoni *et al*.

(2011) while observing a 20% reduction in CH_4 emissions in short-term (10 week) and long-term (2 year) defaunated sheep, found methanogens per gram of DM of rumen content increased with defaunation while the diversity of the dominant methanogenic community was not changed. Therefore, it may not be reasonable to attribute the reduced CH_4 production from defaunation to a loss of methanogens (Morgavi *et al.* 2012).

The presence of protozoa did not change enteric CH_4 production in lambs raised with/without protozoa from birth (Hegarty *et al.* 2008) or from 10 to 25 weeks after chemical defaunation (Bird *et al*. 2008). Defaunation was associated with a reduced number of methanogens in rumen fluid, but did not reduce CH_4 production (Morgavi *et al.* 2012; Kumar *et al.* 2013). This could be explained as defaunation induces changes in bacterial or fungal populations (Eugène *et al.* 2004a) and the absence of protozoa in the rumen leads to changes in the methanogen community (Morgavi *et al.* 2012).

Ruminal acetogens were found able to grow on CO_2 and H_2, and produce acetate, but reductive acetogenesis was not likely to be occurring because of lower H_2 affinity, making reductive acetogenesis unable to compete with methanogens in the rumen (Joblin 1999). In normal fermentation, methanogens reduce H_2 to a low level in which reductive acetogenesis is below detectable levels (Ungerfeld 2015), but if pyruvate-derived acetate is produced when methanogenesis is inhibited, H_2 may accumulate and stimulate reductive acetogenesis (Ungerfeld 2013). Reductive acetogens established in the rumen lacking methanogens can replace methanogens as a sink for H_2 in the rumen (Fonty *et al*. 2007). The reduced CH_4 emissions from defaunated animals associated

with a rise in acetate proportion is a desirable condition in the rumen where reductive acetogens may be occurring.

1.6 Effects of oils or nitrate on rumen fermentation and methane emissions

A variety of strategies to manipulate rumen ecology to reduce enteric CH_4 emissions have been proposed. An immunization approach to control three selected methanogens by vaccination achieved almost 8% reduced CH_4 production in sheep (Wright *et al.* 2004). However, the diverse methanogenic community and the growth of untargeted methanogens may account for the immunization failures (Williams *et al*. 2009). The use of reductive acetogenesis is a potential approach to reduce CH_4 production as acetogens use H_2 to reduce CO_2 to acetate while methanogens reduce CO_2 to CH_4 (Ungerfeld 2013). In the rumen environment acetogens are less numerous and less efficient than methanogens in the competition for H_2 (Joblin 1999), thus results are often unsatisfactory or not conclusive. The use of plant extracts to reduce CH_4 is an increasing interest as a natural alternative, but the use of plant extracts like tannin and saponin has few studies tested on *in vivo* and results are highly variable (Martin *et al*. 2010). The use of essential oils, rich in medium chain fatty acids (MCFA), has shown a promising dietary strategy to reduce CH_4 emissions (Hristov *et al*. 2009; Patra 2014). The reduced CH_4 production by fatty acids is a result of reduced rumen protozoa and methanogens (Matsumoto *et al.* 1991; Dohme *et al.* 2000; Liu *et al.* 2011). Besides inhibitory effects of MCFA on rumen protozoa and methanogensis, addition of oils is also used to increase the dietary energy (Coppock and Wilks 1991). However, palatability of MCFA could reduce animal intake (Lovett et al. 2003; Hollmann et al.

2012) and reduce digestibility of DM and NDF, resulting in a disadvantage of increased oils in diets when targeting a large amount of reduced CH_4 emissions (Patra 2014).

Addition of nitrate (NO_3) in diets has shown a persistent effect of reduced CH_4 emissions from ruminants (van Zijderveld *et al.* 2011; Lee and Beauchemin 2014). Stochiometrically, 1 mol of NO_3 would produce 1 mol of ammonia and should reduce 1 mol or 16g of CH_4 production. However, the potential toxicity of NO_3 in the feed associated with nitrite (NO_2) poisoning constrains its use in practice (Leng and Preston 2010). A sufficient inclusion of NO_3 in diets and a delivery practice is important to reduce CH_4 emissions with no effect on microbial fermentation because NO_2 poisoning occurs only when large quantities of NO_3 in the feed suddenly introduces into rumen without adaptation (Leng and Preston 2010; Lee and Beauchemin 2014). Nitrate is also a major N source and it can be used to replace urea when ruminants are supported on low protein forages (Nolan *et al.* 2010; Li *et al.* 2012).

1.6.1 Oils

The use of oils to kill methanogens and ciliate protozoa in the rumen and reduce CH_4 production has been well reported. Methane production was reduced by 28% and 73% in sheep fed with 3.5% and 7% of coconut oil (CO), but this did not significantly affect energy metabolism or N turnover (Machmüller and Kreuzer 1999). Feeding CO (50g/kg) significantly reduced CH_4 emissions without affecting the total tract digestion or energy retention of the sheep (Machmüller *et al.* 2003). Jordan *et al.* (2006) also showed that feeding 250g/d of refined CO to beef heifers decreased CH_4 output by 18%, but maintained intake and improved animal performance.

A driver of decreased CH_4 output by fatty acids may be the depression in DMI following the CO supplementation (Sutton *et al.* 1983; Machmüller and Kreuzer 1999; Lovett *et al.* 2003; Hollmann *et al.* 2012). This reduction in DMI was probably due to a high level of lauric acid (42%) and myristic acid (15%) in CO. Hristov *et al.* (2011) and Dohme *et al.* (2001) reported that lauric and myristic acid depressed DMI, ruminal fibre degradation and had an adverse effect on ruminal fermentation. Nevertheless, a meta-analysis of effects of dietary fat including sunflower oil, CO, linseed oil and corn oil by Patra (2014) concluded that while ruminal NH_3 concentration and digestibility of DM and NDF were reduced linearly with increasing dietary fat, but total VFA and acetate were not affected. The negative effects of dietary fat on digestibility of DM and NDF were probably accounted for by the decrease in rumen protozoa and fibrolytic bacteria (Patra 2014).

1.6.2 Dietary nitrate

Dietary NO_3 has been shown to reduce CH_4 emissions from ruminants with a consistent and persistent efficacy (Nolan *et al.* 2010; van Zijderveld *et al.* 2010; van Zijderveld *et al.* 2011; de Raphélis-Soissan *et al.* 2014); with a CH_4 reduction range of 23% - 35% when 1.9% to 2.6% NO_3 was supplemented. A review by Leng and Preston (2010) concluded that the use of NO_3 as a H_2 sink could reduce CH_4 production from 16-50%, depending on diets and the inclusion rate of NO_3. This is because approximately 2 moles of H_2 will be needed to convert NO_3 to NO_2 and 6 moles of H_2 will be removed in order to reduce NO_2 to NH_3 (Allison and Reddy 1984). Nitrate causes changes in rumen fermentation, increasing acetate and decreasing propionate proportions as the

high affinity of NO_3 for H_2 is more favourable for NO_2 formation than propionate or CH_4 formation (Ungerfeld and Kohn 2006). This reduces methanogenesis by diminishing H_2 availability (van Zijderveld *et al.* 2010; van Zijderveld *et al.* 2011). Further, shorter mean fluid retention time in the rumen by supplementation of NO_3 is associated with a lower CH_4 yield (Nolan *et al.* 2010). Also, NO_3 can be potentially used to replace urea as a source of NPN for ruminants (Nolan *et al.* 2010; Li *et al.* 2012) and improve microbial N outflow (Guo *et al.* 2009).

1.7 Hypothesis of the research

The majority of the world ruminant animals graze forage that contains insufficient protein for optimal animal production. Elimination of rumen protozoa from ruminants can increase the efficiency of nutrient utilisation, especially of protein. Importantly, this increase in livestock productivity could occur alongside a reduction in CH_4 emissions. Supplementation of fatty acids or dietary NO_3 shows the persistent efficacy of CH_4 mitigation, but these CH_4 mitigations may suppress animal intake and productivity, and NO_3 supplement cannot currently be used as a practical means because of potential risk of nitrite toxicity to the host. Therefore, this research was conducted to test the hypothesis that elimination of rumen protozoa from ruminants and combination of defaunation with fatty acids or NO_3 supplementation could increase the productivity of ruminants, while reducing the amount of CH_4 emitted.

To address the hypothesis, the experimental program conducted is presented in the following order in this thesis: While studies were made in both sheep and cattle, this was not with intent to compare effects of defaunation cross species but simply to use the

most relevant species for each issue considered to be critical to advancing defaunation towards a practical means of improving ruminant productivity.

- Chapter 2 as a baseline study will confirm the efficacy of defaunation to reduce CH_4 emissions and increase productivity of sheep in a controlled feeding environment.
- Chapter 3 will examine the application of defaunation in the grazing environment and determine CH_4 emissions while grazing.
- Chapter 4 will examine the scope of combining defaunation and NO_3 supplementation to enhance productivity and suppress CH_4 emissions of sheep offered low quality roughage.
- Chapters 5 and 6 will examine the effect of defaunation and NO_3 (*in vitro*) and confirm effects of defaunation and fatty acids (*in vivo*) on reducing CH_4 emissions in beef cattle.
- And Chapter 7 in recognition of the difficulty experienced in defaunating cattle, seeks to understand the role of retained protozoa in other stomachs by quantifying the scale of sequestration of protozoa in the rumen, reticulum and omasum.

Chapter 2

Methane emissions, ruminal characteristics and nitrogen utilisation changes after refaunation of protozoa-free sheep

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Abstract

Effects of rumen protozoa on ruminal fermentation, methane (CH_4) emissions and nitrogen (N) retention were studied in twelve crossbred ewes given an oaten chaff diet. Over 10 days sheep were progressively adapted to a diet containing 7% coconut oil distillate to suppress rumen protozoa and then were defaunated using sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol). Twelve weeks after defaunation treatment, five sheep were inoculated with rumen fluid collected from cannulated sheep to refaunate them and the effect of re-establishment of rumen protozoa 0, 7, 14 and 21 days following refaunation on ruminal fermentation and CH_4 emissions was then examined in Experiment 1. As a following study (Experiment 2), feed intake was restricted to 1.5 × ME maintenance from day 28 to day 43 when dry matter digestibility (DMD), N retention, fermentation and daily methane production (DMP) were compared between defaunated and refaunated sheep in a randomised block design. Sheep were scanned by a computed tomography scanner on day 0 and day 28 to estimate reticulo-rumen (RR) weight and carcass composition. It was concluded that refaunated sheep did not have a higher daily DMP than did the defaunated cohort within 21 days after refaunation as measured by GreenFeed Emission Monitoring units. Total volatile fatty acid (VFA) concentration and the proportion of propionate in the rumen VFA gradually increased over 21 days following refaunation (Experiment 1), while a change towards higher butyrate and lower acetate proportions was observed after 28 days (Experiment 2; $P < 0.05$). In experiment 2 feed intake was fixed for comparative studies. There was a tendency towards a heavier RR weight ($P = 0.08$) and a higher ratio of RR to liveweight in defaunated sheep 28 days after refaunation ($P < 0.001$), but carcass composition was

not affected by refaunation status. Experiment 2 showed defaunated sheep had a 7% lower DMP and methane yield (g CH_4/kg DMI) than did refaunated sheep with an established rumen fauna ($P < 0.05$). Apparent whole-tract N digestibility, DMD and microbial crude protein supply were not different between defaunated and refaunated sheep, while energy losses in CH_4 (MJ/d) and CH_4 as a proportion of gross energy intake were both approximately 8% lower in defaunated sheep.

K**eywords:** Ciliates, methanogens, carcass composition.

2.1 Introduction

Rumen protozoa are important, but not essential in the rumen ecosystem and for the well-being of host animals (Williams and Coleman 1992). Elimination of rumen protozoa (defaunation) has led to reported increases in growth rate and average daily gain (ADG) of ruminants (Bird *et al.* 1979; Eugène *et al.* 2004a; Newbold *et al.* 2015) especially when the feed is deficient in protein relative to energy content with respect to the animal's requirements. In addition, rumen protozoa are significant hydrogen (H_2) producers and synthesise mainly acetate and butyrate rather than propionate, so defaunation may be expected to induce a greater proportion of propionate in the ruminal VFA, but this phenomenon is not always observed (Williams and Coleman 1992; Hegarty *et al.* 2008). The reduced daily methane production (DMP) associated with defaunation reported by several authors (Hegarty 1999; Eugène *et al.* 2004a; Newbold *et al.* 2015) may also reflect a reduced ruminal H_2 availability due to (1) reduced acetate and H_2 production, (2) increased H_2 use in propionic acid synthesis, (3) reduced endosymbiotic methanogens associated with rumen protozoa (Finlay *et al.* 1994; Tokura

et al. 1997) and (4) decreased total ruminal dry matter fermentation. Despite these potential actions, defaunation did not change enteric CH_4 production 10 to 25 weeks post-treatment (Bird *et al.* 2008) and did not affect CH_4 production by lambs without protozoa from birth or from weaning (Hegarty *et al.* 2008). The absence of rumen protozoa, therefore, does not always reduce DMP (Morgavi *et al.* 2012; Kumar *et al.* 2013). So, the role of rumen protozoa in moderating the overall H_2 economy and fermentation in the rumen is not consistent or clearly understood. This study aimed to examine the time-course of protozoa establishment, fermentation, intake and CH_4 production changes that occur following refaunation of the rumen (Experiment 1) and then to assess fermentation, CH_4 production, reticulo-rumen characteristics and nitrogen retention and digestibility in defaunated and refaunated sheep with a stable rumen ecology (Experiment 2). This was done to better understand the contribution (direct or indirect) of ciliate protozoa to rumen metabolism and methanogenesis.

2.2 Materials and methods

2.2.1 Preparation of defaunated animals

All protocols for the care and treatment of the sheep were approved by the University of New England Animal Ethics Committee (AEC 14-083). Twelve crossbred ewes (Border Leicester rams × Merino ewes) about 24 months of age with an average liveweight (± s.e) of 55 ± 4 kg were selected and acclimated to a diet of blended lucerne and cereal (LC) chaff for three days while being held in individual pens, so they had no direct physical contact with each other. Sheep were then supplemented with dietary coconut oil distillate (COD; PT Nuansa Kimia Sejati, Tangerang, Indonesia.), from an initial

inclusion of 3% progressing to 7% COD inclusion in the diet (as fed) over 10 days to supress rumen protozoa. The diet of COD was prepared by sprinkling the liquid COD onto LC while the chaff was tossed in a rotary feed mixer. After 10 days of COD inclusion, sheep were fasted and orally dosed with sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol ESB/70AV, Albright and Wilson Australia Ltd, Melbourne) administered at 10 g/day in a 10% v/v solution for three consecutive days to remove protozoa, with feed being withheld during this period. This defaunation protocol was adapted from Bird and Light (2013). Chaff was offered to defaunated sheep 5 hours after the last dose of Empicol. One defaunated sheep was removed from the experiment due to inappetence, but other sheep recovered their pre-treatment voluntary intake within 11 days post-dosing. Small entodiniomorph protozoa were found in some rumen fluid samples 11 days after the treatment completed, so defaunated sheep were again supplemented with 7% COD for 6 days, fasted and dosed for two days with Empicol as before, with feed being withheld during dosing. After the second defaunation treatment, sheep were offered LC and maintained in individual pens for 2 weeks then rumen-samples collected and examined to ensure that rumen protozoa could not be visually detected in any rumen fluid before sheep were moved into 2×2 ha paddocks which excluded their contact with other ruminants (Table 2.1)

After ten weeks of recovery following defaunation treatment, sheep were randomly allocated into 2 groups by stratified randomisation by ranking on liveweight then randomly allocated to treatment within 6 weight classes and housed in two separate group pens in an animal house. Sheep were offered *ad libitum* access to oaten chaff (OC; 9.5 MJ/kg DM and 7.3% CP) delivered by automatic feeders and a restricted pellet

supplement (12 MJ/kg DM and 16.8% CP) was delivered by GreenFeed Emission Monitoring (GEM) units (one GEM per pen; Table 2.2). Radio-frequency identification (RFID) sensors were fitted in both the automatic chaff dispensers and GEM to identify and record individual animal intakes. After 2 week acclimation to the automatic feeders and GEM, Experiment 1 commenced (day 0, Table 2.1).

Table 2.1 Experimental schedule for the defaunation, refaunation and data measurements.

	Day	Activity
Defaunation period	-113	Coconut oil distillate (7% COD) feeding period
	-103	First dosing protocol and recovery period
	-90	COD feeding period between 2 dosing protocol
	-84	Second dosing protocol and recovery period
Recovery period	-71	Defaunated sheep were placed in an isolated paddock for grazing
	-15	Defaunated sheep were allocated to defaunated and refaunated groups and fed oaten chaff in 2 separate group pens. Methane emissions were measured by GEM and intake was measured by auto-feeders.
	-1	Defaunated sheep were scanned to determine RR volumes by CT scan, liveweight was measured before scanning
Refaunation and measurement period	0	Rumen fluid collection for initial measurements. Refaunated 5 sheep with fresh rumen fluid from cannulated sheep for 2 days (50 mL per sheep.day)
	7	Rumen fluid, liveweight and methane emissions from green-feeds were collected every week commencing on d 0, 7, 14 and 21.
	28	Sheep were scanned for the last measurement of RR volumes by CT scan
	29-38	Total collection period
	39-43	Sheep were placed in respiration chambers to measure methane emissions.

Table 2.2 Chemical composition of the pellets supplied through the Greenfeed Emision Monitoring (GEM) unit and of oaten chaff (g/100g dry matter)

Component	GEM pellets	Oaten chaff
Dry matter (in feed as-fed)	90.1	89.5
Dry matter digestibility	75	65
Digestible organic matter	75	62
Inorganic ash	9	7
Organic matter	91	93
Neutral detergent fibre	31	55
Acid detergent fibre	10	29
Crude protein	16.8	7.3
Crude fat	3.9	-
Metabolisable energy (MJ/kg DM)	12.1	9.5

2.2.2 Experiment 1

Starting on day 0, one group of defaunated sheep (n=5) was inoculated (50 mL per sheep per day on 2 consecutive days) with fresh mixed rumen fluid collected from 5 rumen cannulated sheep fed roughage. Samples of rumen fluid (20 mL) were then collected from each sheep weekly by oesophageal intubation with a fresh collection tube used for each animal for protozoal enumeration, volatile fatty acid (VFA) and ammonia (NH_3) analyses. Rumen pH was measured immediately using a portable pH meter (Orion 230 Aplus, Thermo scientific, Beverly, MA, USA). A 15 mL subsample was placed in wide-neck McCartney bottle acidified with 0.25 mL of 18 M sulphuric acid

and stored at -20^{0}C for VFA and NH_3 analyses. A 4 mL subsample was placed in wide-neck McCartney bottle containing 16 mL of formaldehyde-saline (4% formalin v/v) for protozoa enumeration. Protozoa were counted using a Fuchs–Rosenthal optical counting chamber (0.0625 mm^2 and 0.2 mm of depth) using a staining technique adapted from the procedure of Dehority (1984). The protozoa were differentiated into large (>100 µm) and small (<100 µm) holotrich and entodiniomorph groupings. The VFA concentrations were determined by gas chromatography (Nolan *et al.* 2010) using a Varian CP 3800 Gas Chromatograph (Varian Inc. Palo Alto, California USA) and NH_3 concentration was analysed by a modified Berthelot reaction using a continuous flow analyser (San^{++}, Skalar, Breda, The Netherlands).

2.2.3 Methane measurement by GreenFeed Emission Monitoring units

Immediately following refaunation, DMP of all sheep was monitored by the GEM unit present in each pen. For each week, DMP of each sheep was averaged for the 7 days prior to day 0, 7, 14 and 21 and together with the sum of dry matter intake (DMI) in OC and pellets in each period was used to calculate CH_4 yield (MY; g CH_4/kg DMI). For CH_4 to be measured in the GEM, sheep voluntarily placed their heads in a shroud and were detected by the RFID sensors, triggering the GEM to progressively release pellets (Hammond *et al.* 2016). Eructated CH_4 was measured while sheep consumed the pellet supplement. Pellets were dispensed to individual sheep at a minimum of every 4 h/supplementation event (total maximum of 6 supplementation events per day). At each supplementation event, up to 5 drops of pellets were made, with drops being made at 40s intervals and providing 7.74 ± 0.54 g pellets/drop. This supplementation regime

routinely ensured sheep stayed at the GEM for CH_4 and carbon dioxide (CO_2) flux measures for at least 2 min while being supplemented. All visits to the autofeeders and GEM units were continuously recorded from 7 days prior to day 0 and through to day 21. Sheep were also measured for liveweight weekly in the morning without fasting.

2.2.4 Experiment 2

Samples of rumen fluid (20 mL) were collected from each sheep on days 30 and 38 for protozoal enumeration, VFA and NH_3 analyses with sampling procedures as described in Experiment 1. From day 28, sheep were restricted fed at 1.5 × ME maintenance for 4 days before being moved into individual metabolism cages to conduct a 5-day total collection of excreta and then DMP was measured in open circuit respiration chambers. The maintenance requirement (ME_m; MJ ME/d) was calculated from the Australian feeding standards (CSIRO 2007). The reason for restricting feed intake was to allow comparison of DMP and N retention of defaunated and refaunated sheep without confounding by variable daily DMI. The average liveweight of sheep on day 28 was used to calculate ME requirement for all sheep for the restricted feeding period (1.5 M_m) which was an average of 68% of their previous *ad libitum* intake. Feed was divided in two equal portions and offered at 1000 hours and 1500 hours daily during the total collection period. Sheep had free access to fresh water renewed every morning in a water trough.

2.2.5 Estimation of reticulo-rumen weight, gas proportion and carcass composition

On day 0 and 28 after protozoa inoculation, a whole body scan from 3rd - 4th thoracic vertebrae and 1st - 2nd caudal vertebrae with 5 mm thickness, 10 mm spacing and 480 mm field of view was performed using a Picker UltraZ 2000 Computed Tomography (CT) scanner, Philips (Philips Medical Imaging Australia, Sydney, NSW) as described by Kvame and Vangen (2007). After scanning, each CT image was edited using the software program OsiriX (Rosset *et al.* 2004) to estimate reticulo-rumen (RR) volume and then remove non-carcass tissues from each image, leaving carcass fat, lean and bone for estimation of carcass weight (CW) and composition. These images were further divided into tissue areas of fat (-194 to -23), lean (-22 to 146) and bone (147 to 1024) in Houndsfield Units (HU) using the ImageJ software program, which was developed on methods similar to those described by Thompson and Kinghorn (1992) and these were corrected for tissue density to provide estimates of tissue weight based on the relationship between HU and density (Fullerton 1980). Rumen gas volume was calculated by the difference between RR volume estimated by OsiriX with air and estimated by ImageJ without air, and gas proportion was calculated as the gas volume expressed as proportion of the total RR volume.

2.2.6 Nitrogen digestibility, energy utilisation, and microbial protein outflow

A 5-day collection of faecal and urinary output was conducted from day 33 to day 38. All excreta output over the 5 days was collected and weighed, with feed DMI and faecal

DM output used to determine DMD. Dry matter content of feed and faeces were determined by drying samples at 60°C in a fan-forced oven to a constant weight. The concentration of allantoin in the urine was determined colorimetrically (IAEA 1997) using a UV-1201 spectrophotometer (Shimadzu, Japan) reading at 522 nm. The yield of total microbial crude protein (MCP) outflow from the rumen was calculated from allantoin output by using the equations of Chen *et al.* (1992).

Total nitrogen (N) in feed, faeces and urine were determined using an automated Organic Nitrogen Determinator (FP-2000, Leco Corporation, St Joseph, MI). Gross energy (GE) content of feed, faeces and urine were determined using a bomb calorimeter (Calorimeter C7000 with cooling system C7002, IKA Werke, Germany). The energy loss through CH_4 was calculated assuming a CH_4 energy density of 55.6 MJ/kg (Bossel and Eliasson 2003). The ME was determined from the GE consumed less the measured energy loss through faeces, urine and CH_4.

2.2.7 Methane measurement by respiration chambers

Daily methane production was measured by open-circuit respiration chambers from day 39 to day 43 using 2×22 h consecutive periods per sheep (Bird *et al.* 2008). Defaunated sheep were first measured from day 39 to day 41 and refaunated sheep were measured from day 41 to day 43 to avoid the risk of protozoal cross-contamination. Sheep were placed in individual respiration chambers by 1100 hours, with their feed and water already available inside the chambers. The chambers were opened to collect feed refusals, clean faecal trays, and supply fresh feed and water at 0900 hours the following day and then were resealed at 1100 hours. Concentration of CH_4 of air leaving the

chamber was measured by a photoacoustic gas analyser (Innova Model 1312, AirTech Instruments, Ballerup, Denmark). Recovery of CH_4 through the chambers was determined by injection of a known volume of CH_4 and measurement of CH_4 concentration every 2 min for 20 min, with recovery of the dose being calculated by integrating the area under the concentration curve over time.

2.2.8 Statistical analyses

Data was statistically analysed using SAS 9.0 (SAS Institute, Cary, NC). Fermentation parameters, DMI, DMP and liveweight collected in Experiment 1 were subject to repeated-measures analysis of variance in PROC MIXED with protozoal treatment, day and protozoa × day interaction as fixed factors. Fermentation parameters, DMP, whole-tract DMD and N retention collected in Experiment 2 were subject to analysis of variance in PROC GLM. For comparison of RR parameters and carcass composition of defaunated and refaunated sheep on day 28, the model used initial RR parameters and carcass composition on day 0 as a covariate. Protozoa count was log-transformed before statistical analysis. Homogeneity of variance and normal distribution were tested using PROC UNIVARIATE before statistical analysis. Means were analysed using the least squares means (LSMEANS) procedure. A probability of error of less than 5% was considered to be statistically significant.

2.3 Results

2.3.1 Experiment 1

2.3.1.1 Rumen protozoal establishment in refaunated sheep

The concentration of total protozoa in the rumen liquor before defaunation commenced was 17.01 ± 5.88 $\times 10^5$ cells/mL of which small entodiniomorphs accounted for 97% of the total protozoa. Rumen protozoa were reduced by 98% (data not shown) after 10 day supplementation with 7% COD, with holotrichs not visually detected after this time. Sheep became protozoa-free after two programs of treatment with Empicol and stayed free throughout the experiment unless protozoa were introduced for refaunation. The protozoal population in the refaunation inoculum was 11.20 × 10^5 cells/mL with small entodiniomorphs being 80% of the total protozoa. After inoculation, the protozoal population of refaunated sheep reached 12.94×10^5 cells/mL by day 21 which was similar to the protozoal population in the inoculum ($P > 0.05$) before settling to 9.06×10^5 cells/mL on day 30 ($P < 0.05$; Figure 2.1). Small entodiniomorphs were predominant, but decreased from 93% to 80% of the total population from day 7 to 38, respectively while the population of large (> 100 μm) protozoa increased over this time from 4.9% to 18% (Figure 2.1).

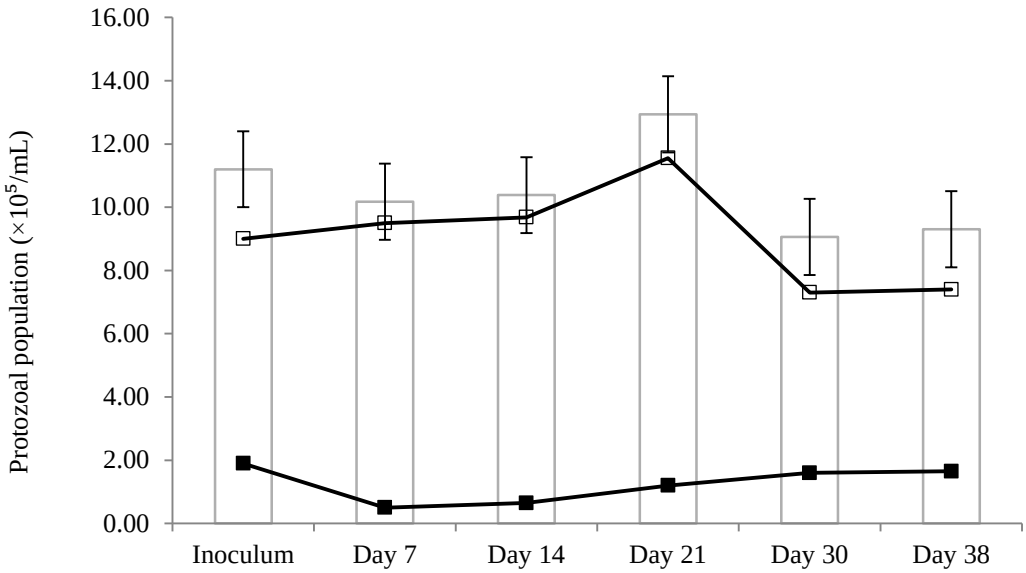

Figure 2.1 Protozoal population of the inoculum and of refaunated sheep on day 7, 14, 21, 30 and 38 after protozoa inoculation. Columns indicate total protozoal population (cells/ml). (□) indicates small entodiniomorphs (< 100 μm). (■) indicates large protozoa (> 100 μm). Error bars indicate pooled s.e of total protozoa. Restricted feed intake occurred after day 21.

2.3.1.2 Ruminal fermentation

Refaunated sheep had higher rumen pH and NH_3 concentration than did defaunated sheep on day 7 and up to day 21 after refaunation ($P < 0.05$; Table 2.3). The introduction of rumen protozoa increased total VFA concentration and the proportion of propionate while the acetate to propionate ratio was lower in refaunated sheep ($P < 0.05$). The proportion of acetate was not affected by the presence or absence of rumen protozoa ($P > 0.05$), but did change with day, being lower 21 days after refaunation while the proportion of butyrate was affected by neither the presence of rumen protozoa nor by day after refaunation ($P > 0.05$).

Table 2.3 Weekly rumen fermentation characteristics, intake and methane emission of defaunated (-P) sheep and of refaunated (+P) sheep before (Day 0) and up to 21 days after protozoa inoculation.

Parameter	-P (n = 6)				+P (n = 5)				Pooled s.e	*P*-value		
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21		Protozoa effect	Day effect	Protozoa × day effect
Liveweight (kg)	54.65	56.08	56.92	57.83	55.18	57.36	57.40	57.86	2.13	0.85	<0.001	0.41
Rumen pH	6.45	6.50	6.33	6.43	6.42	6.62	6.60	6.58	0.05	0.004	0.09	0.15
NH_3-N (mg/L)	55.9	52.38	55.62	50.72	59.54	65.94	62.83	65.24	4.21	0.01	0.98	0.49
Total VFA (mM/L)	90.65	88.29	87.83	88.10	88.49	100.96	103.71	101.88	5.02	0.01	0.49	0.12
Acetate (molar %)	69.80	68.93	67.32	68.26	69.79	68.22	64.35	63.12	1.29	0.10	0.002	0.11
Propionate (molar %)	16.68	16.54	16.92	16.16	16.23	21.80	23.95	23.53	0.82	<0.001	<0.001	0.001
Butyrate (molar %)	9.33	10.31	10.52	10.11	10.53	9.48	10.36	10.98	0.40	0.37	0.25	0.06
Acetate: propionate ratio	4.20	4.20	4.02	4.28	4.36	3.17	2.73	2.73	0.21	0.003	0.004	0.009
Chaff DMI (g/day)	1365	1497	1423	1379	1428	1517	1416	1396	7.25	0.005	0.001	0.009
Pellet DMI (g/day)	162.6	161.2	165.4	163.6	150.1	165.6	181.6	172.9	6.00	0.59	<0.001	0.001
Total DMI (g/day)	1528	1658	1588	1542	1578	1683	1579	1569	8.90	0.01	0.001	0.14
Pellet drops/day	26.71	26.48	27.17	26.88	24.11	26.60	29.17	27.77	0.95	0.94	<0.001	<0.001
DMP (g CH_4/day)[†]	27.83	31.83	33.16	35.17	27.00	31.40	33.60	37.40	1.76	0.88	<0.001	0.06
MY (g CH_4/kg DMI)[†]	18.22	19.19	20.88	22.79	17.12	18.70	21.02	23.84	0.95	0.90	<0.001	0.02

[†]*Daily methane production (DMP) and methane yield (MY) measured by the GEM unit; Dry matter intake (DMI).*

2.3.1.3 Dry matter intake and methane emissions

The presence of rumen protozoa increased DMI (P = 0.01; Table 2.3) with *ad libitum* intake of oaten chaff being higher in refaunated sheep (P < 0.01), while the pellet intake, which was mechanically regulated, was unchanged by refaunation, but was affected by day. Defaunated and refaunated sheep received a similar number of pellet drops per day (P > 0.05) which were close to the maximum allocation of 30 drops per sheep per day, Defaunated and refaunated sheep did not differ in DMP or MY (P > 0.05), but these increased from day 0 to day 21 for both treatments with 21% and 28% increased DMP and 20% and 28% increased MY in defaunated and refaunated sheep, respectively (P < 0.001; Table 2.3). Liveweight did not differ between protozoal treatments (P > 0.05), but increased by 5.5% and 4.6% from day 0 to day 21 in defaunated and refaunated sheep, respectively (P < 0.001; Table 2.3).

2.3.2 Experiment 2

2.3.2.1 Estimation of reticulo-rumen weight and carcass composition

The presence of rumen protozoa 28 days after refaunation tended to decrease RR weight (including tissue and content; P = 0.08) and significantly decreased the ratio of RR to liveweight (P < 0.001), but did not change RR volume, gas volume or proportion of gas space in the RR as estimated by CT scan (P > 0.05; Table 2.4). There was a strong positive correlation between RR weight and RR volume (RR weight = 0.94 RR volume – 0.058; r^2 = 0.96; P < 0.001; Figure 2.2). Carcass weight and weight of fat, lean and

bone 28 days after refaunation were not affected by the presence or absence of rumen protozoa ($P > 0.05$).

Table 2.4 Reticulo-rumen (RR) volume, weight, gas volume and gas proportion of defaunated (- P) sheep and of refaunated (+ P) sheep. Day 0 data was used as a covariate.

Parameter	-P (n = 6)		+P (n = 5)		Pooled s.e	*P*-value
	Day 0[†]	Day 28	Day 0[†]	Day 28		
RR volume (cm^3)	10591	10661	11170	9923	365	0.19
RR weight (kg)	9.75	10.12	10.55	9.16	3.32	0.08
Gas volume (cm^3)	1373	1241	1199	1498	236	0.47
Gas proportion (%)	12.9	11.81	10.78	14.93	2.1	0.33
RR/LW ratio (%)	17.84	17.95	18.99	15.92	0.65	< 0.01
Estimated CW (kg)[‡]	27.11	30.84	26.49	33.88	1.46	0.18
Carcass fat (kg)	6.38	7.96	6.03	8.95	0.61	0.28
Carcass lean (kg)	17.16	19.20	16.96	21.05	0.80	0.14
Carcass bone (kg)	3.57	3.68	3.50	3.89	0.09	0.14
Fat as a % of CW[‡]	23.45	25.51	22.68	26.43	0.83	0.46
Lean as a % of CW[‡]	63.29	62.45	64.11	62.03	0.60	0.64
Bone as a % of CW[‡]	13.26	12.2	13.21	11.58	0.33	0.37

[†]Day 0 data was used as a covariate and statistical comparison were between day 28. [‡]Carcass weight (CW).

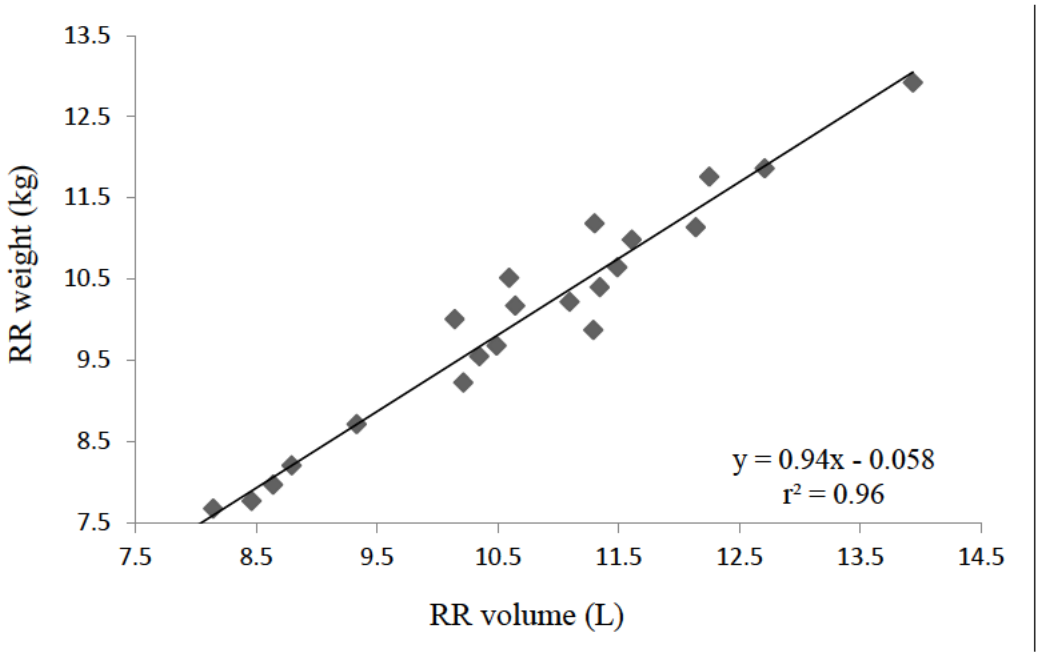

Figure 2.2 Relationship between volume and weigh of rumen-reticulum (RR) with each estimated by Computed Tomography scan.

2.3.2.2 Ruminal fermentation, nitrogen retention and methane emissions

On a uniform restricted feed intake (1.5 ME_m) from day 28 after protozoa inoculation, total VFA concentration did not differ between protozoal treatments ($P > 0.05$; Table 2.5), but was reduced by 37% and 45% compared to the average VFA concentrations on on *ad libitum* intake in defaunated and refaunated sheep respectively. Refaunated sheep continued to exhibit significantly greater NH_3 concentration and proportions of propionate and butyrate ($P < 0.05$) but with a decreased proportion of acetate ($P < 0.01$) on this restricted intake while rumen pH was not affected by refaunation.

Table 2.5 Rumen fermentation characteristics, feed intake, methane emission and nutrient utilisation of defaunated (- P) sheep and of refaunated (+ P) sheep offered a fixed intake (Experiment 2).

Parameter	Protozoal treatment		Difference (-P) - (+P) with pooled s.e	*P*-value
	-P (n=6)	+P (n=5)		
pH	6.45	6.76	-0.31± 0.14	0.17
NH_3-N (mg/L)	44.82	60.37	-15.55 ± 3.61	0.01
Total VFA (mM/L)	56.14	54.19	+1.95 ± 12.13	0.92
Acetate (molar %)	74.70	69.35	+5.35 ± 0.95	0.04
Propionate (molar %)	14.98	16.78	-1.8 ± 0.26	<0.01
Butyrate (molar %)	6.86	9.03	-2.17 ± 0.36	0.002
Acetate/propionate ratio	4.99	4.14	+0.85 ± 0.14	0.001
DMP (g CH_4/day)[†]	19.57	21.03	-1.46 ± 0.47	0.04
MY (g CH_4/kg DMI)[†]	18.44	19.85	-1.41 ± 0.43	0.03
ADG (g/day)[‡]	59.92	57.62	+2.3 ± 14.00	0.91
DMD (%)[§]	59.16	61.21	-2.05 ± 0.95	0.16
Faecal N (g/day)	8.33	8.40	-0.07 ± 0.38	0.90
Urinary N (g/day)	7.45	6.85	+0.60 ± 1.31	0.75
N retention (g/day)	8.91	9.44	-0.53 ± 1.11	0.74
Apparent N digestibility (%)	66.24	65.98	+0.30 ± 1.55	0.91
MCP supply (g/day)[¶]	6.28	5.37	+0.91 ± 0.91	0.49
Energy loss in faeces (MJ/day)	8.178	7.769	+0.41 ± 0.17	0.13
Energy loss in urine (MJ/day)	0.438	0.398	+0.04 ± 0.06	0.66
Energy loss in CH_4 (MJ/day)	1.04	1.13	-0.09 ± 0.03	0.04
Energy loss in CH_4/GE intake (%)	5.61	6.07	-0.46 ± 0.15	0.04
ME (MJ/day)	8.94	9.24	-0.30 ± 0.19	0.22

[†] Daily methane production (DMP) and methane yield (MY) measured by respiration chambers during fix intake period; [‡]Average daily gain (ADG); [§]Dry matter digestibility (DMD); [¶]Microbial crude protein (MCP)

After day 28 when the feed offer was offered at a restricted and uniform daily rate for digestibility and respiration chamber studies, all feed offered was consumed by both defaunated and refaunated sheep with average GE and N intakes of 18.6 MJ/day and 24.7 g N/day (data not shown), respectively. Refaunation did not significantly change the whole-tract DMD, N digestibility, N retention or ME available ($P > 0.05$; Table 2.5). Outflow of MCP was approximately 15% greater in defaunated than refaunated sheep, but there was not a statistical difference ($P > 0.05$). By this time after refaunation, refaunated sheep had a stabilised rumen fermentation with no difference in total VFA concentration, but a greater proportion of propionate and also increased DMP and MY compared to defaunated sheep. Refaunation significantly increased energy loss in CH_4 (MJ/day, $P = 0.04$) as determined by respiration chambers by approximately 8% and increased energy loss as a percentage of GE intake (7.6%; $P = 0.04$) due to the 7% increased DMP with refaunation.

2.4 Discussion

2.4.1 Protozoal population in refaunated sheep after inoculation

The protozoal population in sheep after refaunation from a previously defaunated state was well established by day 7 and reached 12.9×10^5 cells/mL by day 21, comparable with that found by Morgavi *et al.* (2008) who demonstrated that total protozoal populations reached their peak at 12×10^5 cells/mL by 25 to 30 days after inoculation, then stabilised at a slightly reduced population (7.6×10^5 cells/mL). Sénaud *et al.* (1995) also re-inoculated the defaunated rumen with *Isotricha sp*. alone or mixed ciliates and reported that the maximum concentration of rumen protozoa was reached 9 to 17 days

after inoculation. The maximum population then decreased for 2-3 days before stabilising. Further, Zeitz *et al.* (2012) who examined individual growth of *Entodinium caudatum, Epidinium ecaudatum or Eudiplodinium maggii* found that a stabilised population size was reached between 2 and 4 weeks after protozoa inoculation.

2.4.2 Ruminal fermentation and microbial protein outflow

Refaunation increased rumen pH within 21 days of inoculation, which is consistent with previous assessments (Williams and Coleman 1992; Machmüller *et al.* 2003; Eugène *et al.* 2004a), but did not differ from the defaunated group in the restricted feeding period (Newbold *et al.* 2015). Rumen protozoa are able to metabolise lactic acid (Williams and Coleman 1992), so reduce the risk of acidosis associated with a sudden fall in ruminal pH (Jouany and Ushida 1999). Franzolin and Dehority (2010) reported that rumen pH was higher in faunated steers compared to defaunated ones with the mean pH values of 5.98 and 5.50, respectively. These authors also observed that rumen pH was highest before feeding, but lower 4 h after feeding in both defaunated and faunated steers.

The greater proportion of propionate in refaunated sheep by 21 days after inoculation and the higher proportion of propionate, and lower proportion of acetate subsequently observed in refaunated sheep under the restricted feeding condition were inconsistent with previous findings of ciliate protozoa being significant producers of H_2 and synthesising acetic and butyric acids rather than propionic acid (Williams and Coleman 1992). Defaunation is also generally associated with fermentation shifting to a greater proportion of propionic acid, therefore reducing the amount of CH_4 produced (Eugène *et al.* 2004a). However, this phenomenon is not always observed (Williams and

Coleman 1992). A decreased proportion of propionate (Machmüller *et al.* 2003; Hegarty *et al.* 2008) and a higher proportion of acetate in the VFA of defaunated animals has being reported (Bird 1982) when animals were fed roughage based diets. An increased acetate proportion with defaunation may be explained by stoichiometric laws by Sauvant *et al.* (2011), although low digestibility diets typically cause a higher acetate production accompanied by lower ATP yield. A recent meta-analysis by Newbold *et al.* (2015) also found defaunation substantially decreased butyrate and increased acetate, but did not affect propionate proportion.

The finding that ruminants with protozoa (refaunated) supported a higher ruminal NH_3 concentration than did animals with rumen protozoa removed (defaunated) in this study indicated that defaunation may allow a proliferation of rumen bacteria to increase uptake of NH_3 by bacteria for protein synthesis as well as less protein being degraded in the absence of protozoa (Jouany and Ushida 1999). After refaunation, rumen NH_3 concentration increased (Table 2.2) and was 26% higher in refaunated than defaunated sheep after 28 days (Table 2.4), confirming previous assessments (Eugène *et al.* 2004a; Morgavi *et al.* 2012; Newbold *et al.* 2015). Less ruminal catabolism of feed-protein and bacteria occurs in the absence of protozoa, usually leading to an increase in the supply of protein to the duodenum (Bird and Leng 1978; Jouany 1996) and the increased MCP outflow is associated with 9-35% increased ADG in defaunated animals given forage diets (Bird 1989). The present study showed an approximate 15% increase in MCP outflow in defaunated relative to refaunated sheep with a 4% increased ADG (Table 2.4).

2.4.3 Methane emissions

Sequential DMP measures made by the GEM for up to 21 days after protozoa inoculation were not different from those of defaunated sheep. This confirms an earlier study by Morgavi *et al.* (2008) in which refaunated wethers had a similar DMP as defaunated wethers 4 weeks after protozoa inoculation, although protozoal numbers were comparable with conventional animals. Monitoring DMP over 21 days in defaunated and refaunated sheep found that DMP was gradually increased over time in both treatments, indicating that rumen microbes in defaunated sheep had probably not been stabilised after 12 weeks of defaunation treatment and/or the absence of rumen protozoa induced the increased microbial H_2 producers such as cellulolytic ruminococci (Mosoni *et al.* 2011). The increased CH_4 emissions of 7% were observed later in the refaunation treatment at day 43 (Table 2.4). This may be partly explained by the increased proportion of large protozoa in the total protozoal count (Figure 2.1), increasing the butyrate production and H_2 availability for CH_4 formation (Williams and Coleman 1992). Large protozoa also contribute considerable amounts of formate (Tokura *et al.* 1997), which is a substrate for methanogenesis (Leng 2014).

A significant reduction of DMP has been reported in defaunation of animals fed high quality forage or a grain-based diet in association with the increased propionate production (Whitelaw *et al.* 1984; Kreuzer *et al.* 1986; Eugène *et al.* 2004a; Mosoni *et al.* 2011), but differences in DMP were much smaller or even not significant between defaunated and faunated animals on a forage-based diet, on which no increase in

propionate proportion was observed (Hegarty *et al.* 1994; Ranilla *et al.* 2007; Bird *et al.* 2008; Hegarty *et al.* 2008).

The lower DMP by defaunation of animals is probably related to higher concentration of dissolved H_2 due to the reduced capacity to utilise H_2 by rumen microbes (Janssen 2010; Morgavi *et al.* 2012) after removing the endo-symbiotic and ecto-symbiotic methanogens associated with rumen protozoa (Finlay *et al.* 1994; Tokura *et al.* 1997). Ruminal acetogens were found to grow on CO_2 and H_2, and produce acetate, but acetogenesis is generally thought to not occur in the rumen because of the higher H_2 threshold and lower H_2 affinity of acetogens compared to methanogenesis (Joblin 1999). In normal fermentation, methanogens reduce H_2 to a low level in which reductive acetogenesis is below detectable levels (Ungerfeld 2015), but if acetate is produced when methanogenesis is inhibited, H_2 may have accumulated, stimulating reductive acetogenesis (Ungerfeld 2013). Reductive acetogens established in the rumen lacking methanogens can replace methanogens as a sink for H_2 in the rumen (Fonty *et al.* 2007). Although H_2 concentration was not measured in this study to test this hypothesis, the reduced CH_4 emissions from defaunated sheep in this study associated with a rise in acetate proportion is consistent with the accumulation of H_2 resulting from reductive acetogenesis.

2.4.4 Reticulo-rumen weight and carcass composition

An established protozoal population (at 28 d) led to an approximately 10% smaller RR weight and RR weight as a proportion of the liveweight compared to in defaunated sheep. This was consistent with Orpin and Letcher (1984) who reported a 30%

increased rumen volume associated with defaunation. Jouany *et al.* (1988) observed the change in rumen volume following defaunation resulted from changes in ruminal digestion. The increased weight of ruminal contents after defaunation was probably due to longer particle retention of ruminal digesta associated with the rumen fill effect of lower organic matter (OM) digestibility (Eugène *et al.* 2004a). Defaunation of ruminants can increase ADG by 11% because the absence of rumen protozoa allows a compensatory increase in bacterial populations, leading to more microbial protein outflow in defaunated compared to faunated animals (Eugène *et al.* 2004a). There is little data on carcass composition consequences of defaunation with mixed responses reported (Hegarty *et al.* 2000). However, defaunation as a mean to increase protein supply for absorption did not significantly affect body weight or carcass composition of sheep in this study.

2.4.5 Whole-tract dry matter digestibility, nitrogen and energy utilisation

Numerous studies in the literature reported a negative effect of the absence of rumen protozoa on ruminal fibre degradation (Eugène *et al.* 2004a; Eugène *et al.* 2010; Newbold *et al.* 2015), probably due to the loss of protozoal fibrolytic activity. Polysaccharidase activity was greater in the rumen of faunated animals (Santra and Karim 2002; Eugène *et al.* 2004b), resulting in a positive effect of protozoa on ruminal digestion. However, Williams and Withers (1993) observed inoculation with rumen protozoa led to the lowest population of fibrolytic bacteria. Subsequently, major rumen culturable cellulolytic bacterial species such as *Fibrobacter succinogenes*,

Ruminococcus albus and *Ruminococcus flavefaciens* have been shown to exist in increased populations in response to defaunation of the rumen (Mosoni *et al.* 2011; Zeitz *et al.* 2012) due to less competition between bacteria and ciliate protozoa and less predation by protozoa.

Defaunation often induces a shift of N excretion from urine to faeces, but this was not observed in this study. Because the absence of rumen protozoa results in a 5-15% reduction of ruminal digestion of carbohydrate of plant cell walls (Jouany *et al.* 1988) which is compensated for a greater digestion in the large intestine (Ushida *et al.* 1991), more microbial protein is formed in the large intestine, thus the increased faecal N loss is observed (Jouany 1996). In addition, defaunation also causes lower urinary N excretion due to the lower NH_3 concentration in the rumen and less NH_3 absorbed, reducing hepatic urea-synthesis and urinary N loss.

The compensatory increase in cellulolytic bacteria (Mosoni *et al.* 2011) and the greater digestion of carbohydrate of plant cell walls in the large intestine (Ushida *et al.* 1991) that is often associated with the absence of rumen protozoa may explain why no differences between defaunation and refaunation in either the whole-tract DMD nor apparent N digestibility occurred in the present study. Zeitz *et al.* (2012) reported ciliate protozoa did not affect the digestibility of OM, neutral detergent fibre (NDF) or acid detergent fibre (ADF), but apparent N digestibility was increased by the presence of *Eudiplodinium maggii* by 8%. The lack of differences in N excretion through urine and body N retention between defaunated and refaunated sheep was in contrast with Santra *et al.* (2007a) who reported a lower N urinal excretion and greater N retention in

defaunated sheep. The often increased microbial synthesis in defaunated rumen resulted in greater N retention (Bird *et al.* 1994; Jouany 1996), but these were not observed in the present study. Kreuzer *et al.* (1986) reported animals consuming diets rich in fibre increased faecal energy (about 1.26 MJ/day) and urinary energy outputs (about 0.08 MJ/day) than those consuming high starch diets. The urinary energy excretion in the present study was not lower in defaunated sheep, which is in agreement with Kreuzer *et al.* (1986) who fed wethers a diet rich in cellulose content. Reduced energy loss in urine by defaunation is often reported and related to the reduced NH_3 concentration in the rumen, and the increased MCP supply and greater N retention after defaunation (Whitelaw *et al.* 1984; Bird *et al.* 1994; Jouany 1996). A (non-significant) 15 % higher MCP supply in defaunated sheep was observed in this study, while N retention and energy excretion in urine and faeces were not different between treatments. The energy loss in CH_4/GE intake was lower in defaunated than refaunated sheep and these energy loss fell in the range of 5 to 7% as reported by Hristov *et al.* (2013).

2.5 Conclusion

These experiments have confirmed that the absence of protozoa from the rumen leads to an increase in rumen size and has a lower DMP without affecting apparent digestibility. Longer studies are required to quantify ADG and energetic efficiencies, but the present studies confirm the efficacy of defaunation as a strategy to reduce enteric CH_4 emissions of sheep without adverse effects of digestive consequences.

Higher Degree Research Thesis by Publication

University of New England

Statement of Originality

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

Type of work	Paper numbers
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_____ 14 June 2016

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(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Chapter 3

Methane emissions and productivity of defaunated and refaunated sheep while grazing

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Abstract

Rumen protozoa produce hydrogen, which is utilised by methanogens to synthesis enteric methane (CH_4) that is a loss of digested energy and has an adverse environmental impact as a greenhouse gas. The aim of this study was to examine the effect of the absence of rumen protozoa on pasture intake, ruminal fermentation and enteric CH_4 production of grazing sheep. An incomplete crossover experiment was conducted with eleven crossbred ewes (6 without [defaunated] and 5 with protozoa [refaunated]) on 2 × 2 ha pastures with daily CH_4 production (DMP) being measured by GreenFeed Emission Monitoring (GEM) units. It was concluded that grazing defaunated sheep exhibited lower concentration of rumen ammonia (P = 0.01), but similar concentrations of total rumen volatile fatty acids compared to refaunated sheep ($P > 0.05$). The molar proportion of acetate was decreased and butyrate proportion was increased by defaunation, while the proportion of propionate was unchanged by the absence of rumen protozoa. Estimated pasture intake was not different between defaunated and refaunated sheep ($P > 0.05$) Defaunated sheep tended to have a higher total dry matter intake (tDMI; $P = 0.06$), being the sum of pasture intake and pellet supplement intake. There was a tendency towards a lower CH_4 yield (g CH_4/kg tDMI; $P = 0.07$) in defaunated sheep.

Keywords: Pasture intake, defaunation, methane, sheep

3.1 Introduction

Recent meta-analysis confirms that removal of ciliate protozoa from the rumen of ruminants can increase livestock average daily gain by 9% and reduce enteric methane (CH_4) emissions by 11% (Newbold *et al.* 2015). The positive effect of defaunation on animal growth is often seen with poor quality roughage diets that are low in nitrogen content and provide insufficient rumen degradable protein for the growth of rumen microbes (Bird and Leng 1978; Boodoo *et al.* 1978; Williams and Coleman 1992). This may be advantageous in the tropics where forages are often deficient in protein and have higher fibre content than do temperate grasses (Minson 1990). Since higher fibre content is associated with a great CH_4 yield (Margan *et al.* 1988), a higher CH_4 yield as well as reduced animal performance can be expected to coincide. This suggests that elimination of rumen protozoa can improve growth and reduce CH_4 emissions of ruminants grazing on tropical or other low quality pastures. However, there is little data from grazing animals available to confirm this (Bird and Leng 1984; Hegarty *et al.* 2000) and no grazing methane data. This study was conducted to quantify whether effects of defaunation on VFA, DMI, wool growth and CH_4 production observed in controlled feeding studies are also evident in grazing environments.

3.2 Materials and methods

3.2.1 Animals and experimental procedures

The animals and defaunation treatments were described previously in (Chapter 2). Briefly, twelve crossbred ewes (Border Leicester rams × Merino ewes) about 30 months of age, were defaunated by treatment with sodium 1-(2-sulfonatooxyethoxy) dodecane

(Empicol ESB/70AV, Albright and Wilson Australia Ltd, Melbourne) administered at 10 g/day in a 10% v/v solution for three consecutive days. After defaunation one sheep was slow to recover appetite, so was removed from the study. Eighteen weeks after defaunation, 5 sheep were re-inoculated with rumen protozoa and were successfully refaunated for a period of 8 weeks.

Ewes with initial liveweight (± s.e.m) of 56.7 ± 1.9 kg (-P; defaunated) and 57.9 ± 2.0 kg (+P; refaunated) were adapted to the pasture environments in two paddocks adjacent to each other and adjacent to the paddocks to be used in this experiment. After 2 weeks of adaptation to pasture, a 56-day grazing study was conducted with the two groups of sheep managed on 14 day rotation through four paddocks (1 ha paddock, Table 3.1), with a combination of fixed wire and portable electric fence being used to restrict sheep movement. There was a 14 day rest period for each paddock before it was re-grazed in the rotation. The rotation was arranged so that paddock did not bias the estimate of growth or intake by sheep over the 56 day rotational grazing study. Because two full rotations were made (each group grazed each paddock twice), analysis of pasture attributes was analysed for period 1 (rotation 1: d1-28) verses period 2 (rotation 2: day 29-56) and the interaction of period × treatment tested (Table 3.1).

Two GreenFeed Emissions Monitors (GEM) were continuously used to measure CH_4 and carbon dioxide (CO_2) throughout the 56-day study, with the GEM units moved during rotation, so sheep had continuous GEM access. Defaunated sheep were always prevented from physical contact with refaunated sheep or other ruminants by two rows of electric fencing.

Table 3.1 Experimental schedule for pasture rotation and data measurements.

	Day	Activity
	-14	Sheep were shorn and adapted to the pasture environment in 2 paddocks adjacent to the experimental paddocks.
	-4	Rumen fluid for VFA, ammonia and protozoa were collected, liveweight and mid-side patches were measured.
	0-14	Defaunated and refaunated sheep grazed on the experimental paddocks 1 and 3.
	14-28	Defaunated and refaunated sheep grazed on the experimental paddocks 4 and 2
	24-28	Faecal sampling (period 1)
Comparative study	28	Rumen fluid for VFA, ammonia and protozoa were collected and liveweight were measured
	28-42	Defaunated and refaunated sheep grazed on the experimental paddocks 3 and 1
	42-56	Defaunated and refaunated sheep grazed on the experimental paddocks 2 and 4
	49-53	Faecal sampling (period 2)
	56	Rumen fluid for VFA, ammonia and protozoa were collected liveweight, mid-side patches and whole body fleece weight were measured

3.2.2 Estimation of pasture green dry matter

Visual assessment of pasture green dry matter on offer (DM; kg/ha) was conducted on days -4, 17, 31 and 45 using a Crop Circle™ ACS 210 (Holland Scientific, Lincoln NE USA) sensor coupled to a Trimble ProXRS differential receiver and Ranger data-logger. The Crop Circle™ sensor emits NIR (880nm) and red (650nm) light and measures the reflectance coming back from the plant canopy (Lamb *et al.* 2009). The values obtained from the device were calibrated to actual on-ground green DM at the

time of measurement by taking stationary readings of six 30 × 30 cm quadrats. The Normalised Difference Vegetation Index (NDVI) was calculated from the individual light reflectance values (NDVI = ($NIR_{(reflectance)}$ - $Red_{(reflectance)}$)/($NIR_{(reflectance)}$ + $Red_{reflectance)}$) and was correlated with total green DM (kg/ha; Trotter *et al.* 2010). Estimates of green DM for each NDVI were then calculated using the equation below developed over the same paddocks (McPhee *et al.* 2010) with the data being shown in (Figure 3.1).

$$\text{Green dry matter (kg/ha)} = 37.73 \times e^{5.86 \times NDVI} \quad \text{(eq 3.1; McPhee } et\ al. \text{ 2010).}$$

As the standing dead portion of the sample does not contribute to the Crop Circle™ response, it was not measured by the NDVI, but three quadrat pasture samples in each paddock were also cut and dried in a fan-force oven at 60^0 C until constant weight. These pasture samples were further partitioned into green and dead proportions to determine their individual biomass with samples of green and dead pastures analysed for chemical composition using near-infrared spectroscopy (AFIA 2014; Table 3.2).

3.2.3 Predicted dry matter intake and dry matter digestibility

The GrazFeed modelling software (Freer *et al.* 1997) was used to estimate probable pasture dry matter intake (pDMI) for sheep in this study based on the quality and quantity of pasture green and dead DM (Table 3.2) and animal information. GrazFeed also estimated likely pasture selection by sheep, showing selected dry matter digestibility (DMD), crude protein (CP) and metabolisable energy (ME) of pastures (Table 3.3). Total dry matter intake (tDMI) was calculated as the sum of pasture and pellet DMI. This tDMI was then used to calculate CH_4 yield (MY; g CH_4/kg tDMI).

Silica (Si) was chosen as an indigestible marker and dry matter digestibility (DMD) was calculated as follows (Rymer 2000).

$$\text{DMD (\%)} = \left(1 - \frac{\text{Si in pasture DMI}}{\text{Si in faecal DM}}\right) \times 100 \quad \text{(eq. 3.2; Rymer 2000).}$$

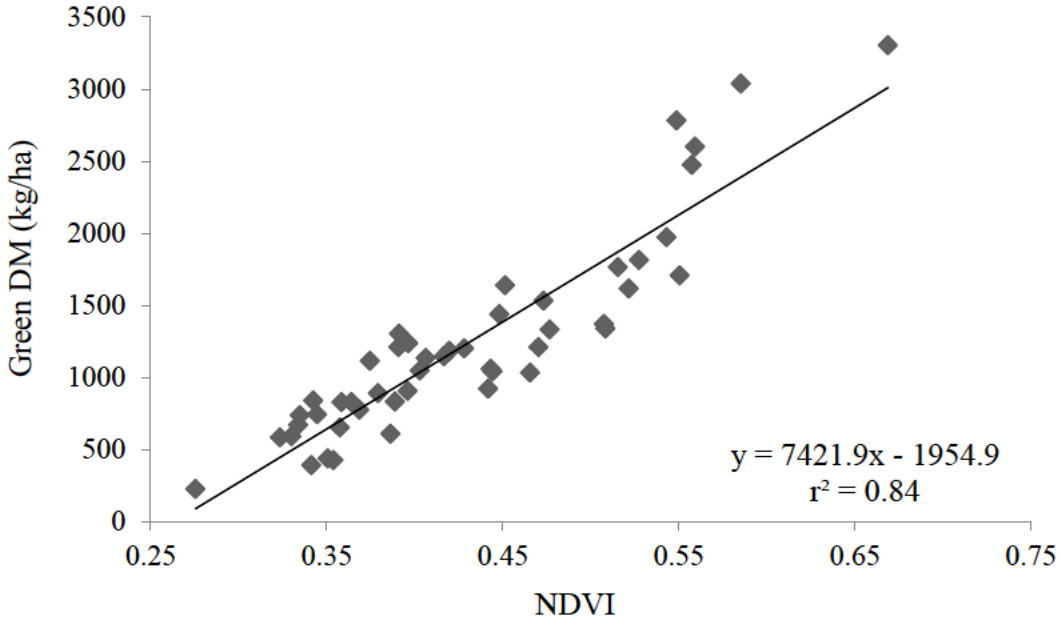

Figure 3.1 The relationship between pasture green dry matter biomass (Green DM; kg DM/ha) and normalised difference vegetation index (NDVI).

Table 3.2 Pasture green dry matter (GDM), pasture green fraction and chemical analysis of the pastures[‡] available to defaunated (-P) and faunated (+P) sheep rotationally grazing. In each 28 day experimental period, sheep had access to two paddocks and all sheep grazed all paddocks over the 56-day study.

Treatment	Period	Paddock number	NDF	ADF	DMD	DOMD	OM	CP	ME	Pasture green DM availability (kg/ha)	Pasture dead DM availability (kg/ha)
-P	1	1	61.3	38.6	55.8	53.5	92.2	9.02	7.99	1319	2132
	1	4	68.0	42.4	52.1	51.2	96.3	6.63	7.41	1095	2419
	2	3	72.9	45.8	46.3	45.1	97.3	5.11	6.04	1350	5154
	2	2	72.2	45.5	50.5	48.7	96.4	5.39	6.77	1285	3705
+P	1	3	71.7	44.9	49.8	47.5	96.9	6.51	6.73	1095	3805
	1	2	67.1	43.1	52.1	50.9	95.0	6.51	7.33	1001	3087
	2	1	64.4	41.1	54.9	54.0	93.6	7.38	7.81	2082	3610
	2	4	73.8	46.0	48.5	47.5	96.5	4.32	6.75	1121	3506
s.e.m			*2.61*	*1.66*	*2.32*	*2.00*	*0.49*	*0.62*	*0.31*	*254*	*1026*
Pasture effect			*0.01*	*0.02*	*0.04*	*0.02*	*<0.01*	*<0.01*	*0.02*	*0.11*	*0.41*
Time effect			*0.06*	*0.06*	*0.16*	*0.19*	*0.03*	*<0.01*	*0.05*	*0.08*	*0.14*
Pasture × time effect			*0.82*	*0.87*	*0.91*	*0.76*	*0.49*	*0.79*	*0.92*	*0.54*	*0.97*

[‡]*Neutral detergent fibre (NDF); acid detergent fibre (ADF); dry matter digestibility (DMD); digestible organic matter in dry matter (DOMD); organic matter (OM); crude protein (CP); metabolisable energy (ME). All are expressed as % in DM while ME is expressed as MJ/kg DM.*

Table 3.3 Crude protein, dry matter digestibility and metabolisable energy content of pastures on offer and that estimated to be selected by defaunated (-P) and faunated (+P) sheep using GrazFeed (Freer *et al.* 1997).

Parameter	-P (n = 6)		+P (n = 5)	
	Period 1	Period 2	Period 1	Period 2
Description of pasture on offer				
Green dry matter (kg/ha)	1207	1318	1048	1602
Dead dry matter (kg/ha)	2276	4429	3446	3558
Dry matter digestibility (DMD; % DM)	52	51	48	52
Crude protein (CP; % DM)	7.8	5.3	6.5	6.0
Metabolisable energy (MJ/kg DM)	7.7	6.4	7.0	7.3
Description of pasture selected (GrazFeed estimate)				
Pasture selected DMD (% DM)	77	78	76	77
Pasture selected CP (% DM)	13	14	13	11
Pasture selected ME (MJ/kg DM)	11.1	11.2	10.9	11.2

3.2.4 Methane and carbon dioxide measurement by Greenfeed Emission Monitoring units

Daily production of methane and CO_2 of all sheep was measured by the GEM units present with each group. A restricted pellet supplement (12 MJ/kg DM and 15% CP, Table 3.4) with chromium oxide (Cr_2O_3) inclusion (1.46 mg Cr_2O_3/kg pellet mix) was mechanically delivered by the GEM units. Sheep voluntarily placed their heads in the

GEM shroud and were detected by the radio-frequency identification sensor, triggering the GEM to progressively release pellets (Hammond *et al.* 2016). Eructated CH_4 and CO_2 were measured while sheep consumed the pellet supplement. Pellets were dispensed to individual sheep no more frequently than every 4h (total maximum of 5 supplementation events per day). At each supplementation event, up to 5 drops of pellets were made, with drops being made at 40s intervals and providing 7.5 ± 0.41 g pellets/drop. This supplementation regime routinely encouraged sheep at the GEM for CH_4 and CO_2 measures for at least 2 min while being supplemented.

Table 3.4 Chemical composition of the pellets supplied through the GreenFeed Emision Monitoring (GEM) unit (g/100g dry matter).

Component	GEM Pellets
Dry matter (in feed as-fed)	92.2
Dry matter digestibility	72
Digestible organic matter	71
Inorganic ash	8
Organic matter	92
Neutral detergent fibre	38
Acid detergent fibre	11
Crude protein	15.2
Crude fat	5.5
Metabolisable energy (MJ/kg DM)	12.2

3.2.5 Analytical procedures

Faecal samples collected from the rectum of all sheep on days 24-28 (period 1) and days 49-53 (period 2), were stored frozen and later dried to constant weight at 60^0C in a fan-force oven, ground through a 1-mm screen and concentrations of chromium and silica (Si in feed and faeces; Barnett *et al.* 2016) were determined using a portable X-ray fluorescence spectroscopy (Bruker Tracer III-V pXRF, Bruker Corp, MA USA). Samples of rumen fluid (20 mL) were collected from each sheep on day 28 and day 56 by oesophageal intubation (using a fresh collection tube used for each animal) for protozoal enumeration, VFA and NH_3 analyses. Rumen pH was measured immediately using a portable pH meter (Orion 230 Aplus, Thermo scientific, Beverly, MA, USA). A 15 mL subsample was placed in wide-neck McCartney bottle acidified with 0.25 mL of 18 M sulphuric acid and stored at -20^0C for VFA and NH_3 analyses. A 4 mL subsample was placed in a wide-neck McCartney bottle containing 16 mL of formaldehyde-saline (4% formalin v/v) for protozoa enumeration. Protozoa were subsequently counted using a Fuchs–Rosenthal optical counting chamber (0.0625 mm^2, 0.2 mm of depth) using a staining technique adapted from Dehority (1984). The protozoa were differentiated into large (>100 µm) and small (<100 µm) holotrich and entodiniomorph groupings. The VFA concentrations were determined by gas chromatography (Nolan *et al.* 2010) using a Varian CP 3800 Gas Chromatograph (Varian Inc. Palo Alto, California USA) and NH_3 concentration was analysed by a modified Berthelot reaction using a continuous flow analyser (San^{++}, Skalar, Breda, The Netherlands).

Sheep were weighed on days -4, 28 and 56. Clean wool growth rate (CWG), wool yield, and fibre diameter were determined on the mid-side of the sheep from day -4 to day 56

by clipping a patch 10 × 10 cm (Oster Golden A5 clippers, blade size 30 model, Cryogen X, USA). After the wool from the patch was clipped, four sides and one diagonal were measured and the area of the patch was calculated using Heron's formula (De Barbieri et al. 2014). Wool samples were sent to New England Fibre Testing (Pty Limited, Walcha NSW Australia) to determine yield and fibre diameter. Sheep were shorn on days -14 and 56 to measure greasy fleece weight.

3.2.6 Statistical analyses

Because both groups of sheep were rotated across all paddocks (twice) over the 56 days, the role of paddock was accounted for in experimental design and so paddock was not included in statistical models. However effect of time on pasture growth was assessed by comparison of means over the first rotation (d1-d28) with the second rotation (d29-56). Rumen fermentation parameters, DMP, MY, DMI and DMD were analysed using the repeated-measures analysis of variance using SAS 9.0 (SAS Institute, Cary, NC) with protozoal treatment, day and protozoa × day interaction as fixed factors. Final liveweight, ADG, CWG, greasy fleece weight and wool fibre diameter were subject to analysis of variance. Effects of unbalanced numbers between treatments was not accounted for. Means were analysed using the least squares means (LSMEANS) procedure. A probability of error of less than 5% was considered to be statistically significant.

3.3 Results

3.3.1 Pastures

The availability of green and dead DM and pasture quality are shown in Table 3.2. The paddocks did not have a significant difference in green or dead biomass availability ($P > 0.05$), but the pasture green DM tended to increase in biomass from period 1 to period 2 ($P = 0.08$). The chemical composition of pastures in the paddocks was different ($P < 0.05$) and was affected by period. Pasture NDF and ADF tended to increase ($P = 0.06$) between period 1 (67.03% and 42.25%, respectively) and period 2 (70.83% and 44.60%, respectively) while CP and ME significantly decreased ($P < 0.05$) between period 1 (7.15% and 7.37 MJ/kg, respectively) and period 2 (5.55% and 6.84MJ/kg, respectively). Dry matter digestibility and DOMD did not differ between two periods ($P > 0.05$).

The chemical composition of selected pasture as estimated by GrazFeed showed a 35% higher DMD and ME and a 49.8% higher CP than that of the average composition of pasture biomass available (Table 3.3).

3.3.2 Ruminal fermentation and methane production

Defaunated sheep had a lower rumen pH and concentration of rumen NH_3 ($P = 0.01$) than did refaunated sheep and the concentration of rumen NH_3 was higher in period 2 than period 1 in both groups of sheep ($P = 0.01$; Table 3.5). The total concentration of rumen VFA was not affected by either protozoa or period ($P > 0.05$) but there was a shift of molar VFA proportion to a reduced proportion of acetate and increased

proportion of butyrate ($P < 0.05$) in defaunated sheep while DMP was not different between defaunated and refaunated sheep ($P > 0.05$). The MY tended to be lower in defaunated than refaunated sheep ($P = 0.06$), with pasture DMI used to calculate MY being estimated by GrazFeed. The DMP increased between period 1 and period 2 in both defaunated (7%) and refaunated sheep (11%; $P = 0.04$) in keeping with greater pasture biomass availability in period 2. The intake of pellets from GEM units differed between groups reflecting fewer visits to the GEM and so a lower voluntary intake of these pellets by the refaunated sheep (Table 3.5).

3.3.3 Dry matter intake, liveweight gain and wool production

Pellet DMI, which was mechanically regulated by the GEM, was higher in defaunated than refaunated sheep ($P < 0.05$; Table 3.5). Concentrations of Si and Cr in feed and faeces were determined, but daily Cr intake of individuals was not constant due to highly variable consumption of GEM pellets, making it difficult to have a plateau level in the faeces to estimate pasture intake and Cr data was not used further. Pasture DMI (pDMI) was, therefore, estimated by GrazFeed and it was estimated to be not affected by protozoa or period ($P > 0.05$). Total DMI (tDMI) tended to be greater in defaunated than refaunated sheep ($P = 0.06$) due to greater pellet DMI by defaunated than refaunated sheep ($P = 0.03$). Estimated DMD was not different between defaunated and refaunated sheep ($P > 0.05$) with no difference in Si concentration in faecal DM (Table 3.5). Average daily gain was not affected by protozoa or period ($P > 0.05$) and there were no differences in CWG, greasy fleece weight or wool fibre diameter between defaunated and refaunated sheep in the grazing environment ($P > 0.05$; Table 3.6).

Table 3.5 Intake, rumen fermentation parameters and methane emissions of defaunated (-P) and refaunated sheep (+P) grazing pastures.

Parameter	-P (n = 6)		+P (n = 5)		Pooled s.e	*P*-value		
	Period 1	Period 2	Period 1	Period 2		Protozoal effect	Day effect	Protozoa × day effect
pH	6.38	6.65	6.90	6.54	0.08	0.01	0.60	0.04
NH_3-N (mg/L)	33.98	49.46	51.36	62.76	4.87	0.01	0.01	0.68
Total VFA (mM/L)	88.51	92.20	99.58	98.95	8.42	0.91	0.14	0.29
Acetate (molar %)	60.84	61.56	62.93	64.89	1.15	0.03	0.26	0.59
Propionate (molar %)	22.84	22.45	23.90	20.77	0.69	0.62	0.02	0.06
Butyrate (molar %)	14.23	13.83	11.40	11.03	1.14	0.02	0.74	0.99
Acetate/propionate	2.68	2.77	2.64	3.13	0.12	0.19	0.02	0.10
Faecal silica (mg/kg DM)	5.43	4.77	5.36	3.27	0.92	0.41	0.16	0.45
DMD[*]	59.51	58.62	55.46	57.32	8.58	0.76	0.95	0.87
DMP (g CH_4/day)[†]	27.52	29.61	27.78	31.21	1.50	0.54	0.04	0.62
pDMI (kg/day)	1.167	1.224	1.170	1.099	0.04	0.12	0.85	0.10
tDMI (kg/day)[5]	1.246	1.306	1.239	1.165	0.03	0.06	0.85	0.08
MY (g CH_4/kg tDMI)[5]	22.09	22.75	22.47	26.72	1.09	0.06	0.04	0.12
Pellet DMI (g/day)	79.6	82.6	68.9	66.2	5.73	0.03	0.98	0.63
Pellet drops/day	11.05	11.47	9.58	9.20	0.79	0.04	0.89	0.62
Total protozoa ($\times 10^5$ cells/ml)	0	0	15.56	18.21	6.66		0.17	

[*]Dry matter digestibility (DMD) was estimated by silica marker (eq.3.2); [†] Daily methane production (DMP) was measured by Greenfeed Emission Monitor; [5]Total dry matter intake (tDMI; kg/d) was the sum of estimated pasture DMI by Grazfeed and pellet DMI measured by GEM. Methane yield (MY) was calculated as DMP divided by tDMI.

Table 3.6 Wool parameters of defaunated (-P) and refaunated sheep (+P) grazing pastures.

Parameter	Protozoal treatment		Difference (-P) - (+P) with pooled s.d	*P*-value
	-P (n=6)	+P (n=5)		
Final liveweight (kg)	68.32	68.72	-0.4 ± 1.04	0.93
Average daily gain (g/day)	187.5	181.2	+6.3 ± 16.2	0.79
Clean wool growth (μg/cm^3/d)	724.3	747.2	-22.9 ± 119	0.77
Greasy fleece weight (kg)	1.30	1.27	+0.03 ± 0.21	0.54
Wool fibre diameter (μm)	30.42	29.68	+0.74 ± 3.42	0.75

3.4 Discussion

3.4.1 Animal productivity

While pasture DMI and DMD were not measured directly in this study; pDMI was estimated by GrezFeed and DMD by Si concentration in pasture and faecal DM and no protozoa effects on these were observed. Lower DMI and DMD in defaunated ruminants are often reported in the literature (Newbold *et al.* 2015), probably due to the loss of protozoal fibrolytic activity and longer particle retention of ruminal digesta associated with the slower rumen outflow and greater rumen fill associated with lower ruminal DMD (Eugène *et al*. 2004a). The lack of differences in DMI and DMD due to protozoa indicates that the only scope for protozoa to affect animal performance in this study would have been through changing the nutrients array or quantity from the fermentation and microbial growth processes. Considering the lower rumen NH_3 concentration in defaunated sheep, it is likely that defaunated sheep did have lower

rumen proteolysis as defaunation often increases microbial protein outflow (Newbold *et al.* 2015). It was unexpected that defaunated sheep did not have greater wool growth response than refaunated sheep consuming pasture as wool growth is responsive to amino acid supply (Reis *et al.* 1973). Certainly, wool growth of crossbred ewes used in this study is less than from Merino sheep which have been often shown responses following defaunation (Bird and Leng 1984), and this low wool growth potential may be why a wool growth response of defaunated sheep in the current study was not apparent.

The lack of effect of protozoa on ADG and wool production on this low protein pasture is in contrast to the literature. Bird and Leng (1984) observed a 23% greater rate of ADG and a 19% greater wool growth rate in defaunated compared to faunated lambs grazed on a green oats forage. Protozoa-free lambs born from defaunated ewes were also 4-8% heavier than were lambs born from faunated ewes and wool growth was greater in protozoa-free lambs grazed on fescue dominant pastures (Hegarty *et al.* 2000). Sheep in the current study, though more mature than those of Bird and Leng (1984), were still having a high protein demand for growth to support an ADG of almost 200 g/day. Hence, it is again surprising that defaunation did not cause a significant difference in ADG. The concentration of NH_3 in the rumen of defaunated sheep was below 50 mg/L which is below microbial protein yield required for the optimal growth of rumen bacteria (Satter and Slyter 1974). However, both microbial protein outflow and dietary protein intake by sheep need to be considered in explaining why ADG and wool growth following defaunation was not increased by defaunation as expected in the grazing environment.

3.4.2 Rumen fermentation and daily methane production

The reduced rumen pH in defaunated sheep confirms that rumen protozoa may have a role in stabilising rumen pH and avoiding the risk of acidosis associated with sudden fall in pH (Jouany and Ushida 1999), but this is not often seen (Newbold *et al.* 2015) and acidosis would have be unlikely on the fibrous pastures. The reduced concentration of rumen NH_3 following defaunation, however, is consistent between the current study and in the literature (Newbold *et al.* 2015). This is due to the reduced deamination of bacterial protein through protozoa predation and less feed-protein being degraded in the rumen of the absence of protozoa (Williams and Coleman 1992; Jouany and Ushida 1999).

A reduced concentration of total VFA following defaunation is often reported (Newbold *et al.* 2015) being ascribed to (i) reduced ruminal DM fermentation (Eugène *et al.* 2004a; Newbold *et al.* 2015) (ii) increased fermented materials being captured in rumen microbial cells rather than producing VFA for the host (Williams and Coleman 1992) and (iii) a larger rumen volume, but reduced total VFA concentration was not induced by defaunation in the current study. This is consistent with no difference in faecal Si, suggesting neither ruminal nor whole tract digestibility were affected by protozoa. The reduced proportion of acetate following defaunation is consistent with Eugène *et al.* (2004a). Differences in VFA proportions after defaunation are often seen as an increased proportion of propionate and a decreased proportion of acetate (Eugène *et al.* 2004a) due to removing rumen protozoa which synthesise acetic acid rather than propionic acid (Williams and Coleman 1992), therefore reducing hydrogen availability

to methanogens and the amount of CH_4 produced. The butyrate content was unusually high, and the higher proportion of butyrate in the rumen VFA from defaunated sheep relative to refaunated sheep in the current study is inconsistent with Williams and Coleman (1992) or Newbold *et al.* (2015).

The lack of effect on DMP due to defaunation in this study is inconsistent with housed studies where defaunation is often associated with average 11 to 13% CH_4 mitigation (Hegarty 1999; Newbold *et al.* 2015). However, the mechanism through which DMP is reduced by defaunation is not well understood (Newbold *et al.* 2015). Defaunation decreases methanogens existing as endo and ecto-symbionts with rumen protozoa (Finlay *et al.* 1994; Tokura *et al.* 1997; Kumar *et al.* 2013), but CH_4 emissions are not always decreased (Kumar *et al.* 2013). This may be due to the complexity of multiple rumen microbes being involved following defaunation (Morgavi *et al.* 2010). Hence, reduced CH_4 emissions following defaunation is not always a consistent consequence of the decreased symbiotic habitat of methanogens (Morgavi *et al.* 2012). The loss of the cilate-associated methanogens in defaunated animals may have been compensated for by an increase in population of cellulolytic ruminococci which are large hydrogen producers (Mosoni *et al.* 2011). The changes in the methanogenic population following defaunation are inconsistent among studies (McAllister and Newbold 2008; Mosoni *et al.* 2011; Morgavi *et al.* 2012; Kumar *et al.* 2013). Therefore, our understanding in the relationship between rumen ciliate-associated methanogens and CH_4 production in defaunated animals is still limited.

3.5 Conclusion

This study reported for the first time that MY tended to be lower in protozoa-free sheep relative to refaunated sheep in the grazing environment, though it is acknowledged that pasture intake used in calculating MY was estimated from modelling. An accurate measure of pasture intake, therefore, is required to confirm this finding. The lack of animal performance (ADG and CWG) responses to defaunation, together with lack of effect on DMP suggest the need for caution in assuming defaunation will improve the productivity and reduce the environmental impact of grazing ruminants.

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Chapter 4

Use of dietary nitrate to increase productivity and reduce methane production of defaunated and faunated lambs consuming protein deficient chaff

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Abstract

The effects of dietary nitrate supplementation and defaunation on methane (CH_4) emissions, microbial protein outflow, digesta kinetics and average daily gain were studied in lambs fed chaff containing 4.1% crude protein in dry matter. Twenty ewe lambs were randomly allocated in a 2×2 factorial experiment (0 or 2% nitrate supplementation and defaunated or faunated protozoal state). Nitrate supplementation increased blood methaemoglobin concentration ($P < 0.05$), rumen volatile fatty acid and ammonia concentrations, dry matter intake, microbial protein outflow, average daily gain, dry matter digestibility, clean wool growth and wool fibre diameter ($P < 0.01$). Nitrate increased CH_4 production (g/d) due to greater dry matter intake, but did not affect CH_4 yield (g CH_4/kg dry matter intake). Nitrate-supplemented lambs had a shorter total mean retention time of digesta in the gut ($P < 0.05$). Defaunation reduced CH_4 production and CH_4 yield by 43 and 47%, but did not cause changes in dry matter intake, microbial protein outflow, average daily gain or clean wool growth. Defaunation decreased total volatile fatty acids and the molar percentage of propionate, but increased the molar percentage of acetate ($P < 0.05$). Interactions were observed such that combined treatments of defaunation and nitrate supplementation increased blood methaemoglobin ($P = 0.04$), and decreased CH_4 yield ($P = 0.01$) relative to other treatment combinations.

Keywords: Methanogensis, protozoa, methane, sheep

4.1 Introduction

Residues of crops and agricultural by-products are the major feed sources for livestock in tropical and subtropical regions, but they are often of low to moderate digestibility with low levels of protein and minerals (Preston 1995). Ruminant production from these low-quality feeds is limited by the deficiency of absorbed amino acids and energy, resulting from decreased feed intake and digestibility due to slow growth and fermentation by rumen microbes (Leng 1990).

Removal of protozoa from the rumen (defaunation) increases average daily gain by 11% (Eugène *et al.* 2004a) due to increased bacterial biomass and increased availability of protein at the duodenum (Bird and Leng 1978; Jouany 1996). Defaunated cattle grew at a 43% greater rate than faunated cattle on the same intake of a low-protein molasses based diet (Bird and Leng 1978) while defaunated lambs showed increased growth rate and wool growth on a low-protein diet (Bird *et al.* 1979). Defaunation also decreases enteric methane (CH_4) production (Hegarty 1999; Newbold *et al.* 2015) by eliminating methanogens that exist as endo- and ecto-symbionts with ciliate protozoa (Finlay *et al.* 1994) and by often changing the molar proportions of volatile fatty acids to a greater proportion of propionate and less proportion of butyrate (Eugène *et al.* 2004a). In constrast, studies of isolated lambs raised without protozoa from birth and defaunated ewes had been shown that absence of rumen protozoa did not reduce CH_4 production (Bird *et al.* 2008; Hegarty *et al.* 2008). Therefore, there is a lack of certainty whether CH_4 emissions are decreased by defaunation.

Dietary nitrate has shown potential to decrease CH_4 emissions from ruminants with a consistent and persistent efficacy (Guo *et al.* 2009; Nolan *et al.* 2010; van Zijderveld *et al.* 2010; van Zijderveld *et al.* 2011; Lee and Beauchemin 2014). This is because hydrogen (H_2) is used by microbes to reduce carbon dioxide (CO_2) to CH_4 (Nolan 1999), but when nitrate is present in the rumen, approximately 2 moles of H_2 will be needed to convert one mole of nitrate to nitrite and 6 moles of H_2 will be required in order to reduce this nitrite to ammonia (Allison and Reddy 1984). A review by Leng and Preston (2010) concluded that the use of nitrate as a H_2 sink could theoretically reduce CH_4 production by 16-50%, depending on diet and the inclusion rate of nitrate, but there is little data on productivity of nitrate-supplemented ruminants on protein deficient roughage. Previous studies have largely focused on a comparison of nitrate and urea as non-protein nitrogen (NPN) sources for ruminant diets and on reducing CH_4 production (Nolan *et al.* 2010; Li *et al.* 2012; de Raphélis-Soissan *et al.* 2014). Despite these potential benefits of nitrate supplements, little is known about the effects of nitrate on microbial fermentation and growth in the rumen without protozoa, especially in animals offered diets unbalanced for nitrogen and energy. This experiment aimed to quantify the effects of nitrate as a source of NPN and the interaction with defaunation on CH_4 production and productivity of lambs offered a protein deficient chaff diet.

4.2 Materials and methods

4.2.1 Animals and feeding

All protocols for care and treatment of the sheep were approved by the University of New England Animal Ethics Committee (AEC 14-083). Merino ewe lambs (n = 20; 38

± 1.9 kg; 13 months of age) were selected and acclimated to a diet of oaten chaff. Lambs were allocated to dietary nitrogen (N) levels by stratified randomisation based on liveweight. The experiment was a 2×2 factorial design (calcium nitrate supplementation at 0 or 3.1%; protozoa status either defaunated or faunated). The diet of 3.1% calcium nitrate (~2% NO_3 as $5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway) was prepared by sprinkling a dilute solution of the nitrate onto oaten chaff while the chaff was tossed in a rotary feed mixer (+NO_3 ; Table 4.1). Another diet (control) was only oaten chaff (-NO_3; Table 4.1).

The experiment lasted for 93 days. Lambs were gradually adapted to nitrate-supplemented oaten chaff from day 0 to day 15 from an initial inclusion of NO_3 of 1% up to 2%, with the dose of calcium NO_3 increased every two days. After this period of NO_3 adaptation, lambs were given *ad libitum* access to nitrate-supplemented oaten chaff with 2% NO_3 from day 16 to day 40. Lambs were placed on restricted intake (80% individual *ad libitum* intake) 5 days before entering respiration chambers on day 45 to day 50 and continued receiving restricted intake from day 50 to day 59 for study of digesta kinetics and total excreta collection and from day 59 to day 64 for repeated measure of CH_4 emissions in respiration chambers. Lambs resumed *ad libitum* intake on day 64 until the end of the experiment. Lambs were fed twice daily in two equal portions at 0930 and 1500 hours. Water was available at all times.

Table 4.1 Chemical composition of the oaten chaff and nitrate-supplemented chaff (g/100g dry matter).

Component	Oaten chaff ($-NO_3$)	Oaten chaff ($+NO_3$) (3.1% Bolifor)
Dry matter (g/100g as fed)	90.2	89.6
Dry matter digestibility	71	70
Digestible organic matter	67	66
Inorganic ash	6.4	7.3
Organic matter	93.6	92.7
Neutral detergent fibre	49	48
Acid detergent fibre	26	25
Crude protein	4.1	7.1
Metabolisable energy (MJ/kg)	10.6	10.4
Nitrate-nitrogen (mg/kg)	60.3	4,300
Nitrate	0.03	1.9

Bolifor CNF: $5Ca(NO_3)_2.NH_4NO_3.10H_2O$ (63.12% nitrate in Bolifor CNF)

4.2.2 Feed sampling and chemical analyses

Samples of oaten chaff (100 g) were collected before and after each mix of feed and stored in -20^0 C. All samples were pooled and sub-samples were taken to analyse for chemical composition (Table 4.1). Feed samples were analysed by the NSW DPI Feed Quality Service, Wagga Wagga Agriculture Institute, NSW, Australia. Feed dry matter, crude protein, acid detergent fibre, neutral detergent fibre, and inorganic ash were determined by wet chemistry. Feed dry matter digestibility and digestible organic matter were determined by near-infrared spectroscopy (AFIA 2014).

4.2.3 Defaunation of lambs

Ten lambs were offered lucerne cereal mix supplemented with coconut oil distillate (COD) with initial inclusion of 3% to 5% of COD over 7 days to supress rumen protozoa. After 7 days feeding COD, lambs were fasted for 24h and orally dosed with sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol ESB/70AV, Albright and Wilson Australia Ltd, Melbourne) administered at 10 g/d in a 10% v/v solution for three consecutive days to remove protozoa. Feed was withheld during this period. Animals required 12 days to recover to their pre-treatment voluntary intake and the three day dosing with Empicol was then repeated. Defaunated lambs were offered lucerne cereal mix during second drenching period and a further 14 days after the second drenching program to recover from defaunation treatment. Fourteen days after second drenching, defaunated and faunated lambs were given *ad libitum* access to oaten chaff for 7 days before day 0. During the defaunation period, the 10 faunated lambs were restricted fed at their maintenance requirement (CSIRO 2007) to prevent divergence in liveweight while the defaunated group was being prepared.

4.2.4 Blood methaemoglobin

Blood was sampled between 2.5 and 3 h after morning feeding on days 0, 15, 50 and 85. A sample of 8 mL was taken from a jugular vein, using lithium heparinised vacutainers (BD Franklin Lakes, NJ, USA). Whole blood methaemoglobin (MetHb) concentration was determined within 30 min using a blood gas analyser (ABL 800 Flex, Radiometer, BrØnshØj, Denmark).

4.2.5 Methane production

Daily CH_4 production (DMP; g CH_4/day) of each lamb was measured in open-circuit respiration chambers over 2×22-h consecutive periods (Bird *et al.* 2008). Lambs were placed in individual respiration chambers by 1100 hours, with their feed and water available inside the chambers. The chambers were opened to collect feed refusals, clean faecal trays, and supply fresh feed and water at 0900 hours the following day and then were resealed at 1100 hours.

Sub-samples of air within each chamber and of the ambient air were collected every 13 min into Tedlar gas sampling bags (Supelco, Bellefonte, PA, USA) continuously over the 22 h of confinement for analysis. Methane concentration was measured by a photoacoustic gas analyser (Innova Model 1312, AirTech Instruments, Ballerup, Denmark). Recovery of CH_4 through the chambers was determined by injection of a known volume of CH_4 and measurement of CH_4 concentration every 2 min for 20 min, with recovery of the dose being calculated by integrating the area under the concentration curve over time.

4.2.6 Digestibility, digesta kinetics and microbial protein outflow

Lambs were placed in metabolism cages and a 6-day collection of faecal and urinary output was conducted. All faecal output over the 6 days was collected and weighed with feed DMI and faecal DM output used to determine dry matter digestibility (DMD). Concurrent with determining DMD, the mean retention time (MRT) of digesta was estimated in all lambs over 6 days by reference to faecal excretion of a dosed particle-

phase marker (5 g per lamb of Cr-mordanted NDF from oaten chaff) and liquid-phase marker (5 g per lamb of CoEDTA from AVA Chemicals Pty Ltd. Mumbai, India in 45mL of Milli-Q water). The non-digestible Cr-mordanted NDF was prepared in accordance with Udén *et al.* (1980) and with CoEDTA administered via intubation directly into the rumen as a single dose at 10:00 hours on day 53. Faecal samples were collected every 2 h for the first 24 h, starting 8 h after marker administration, then every 4 h for the next 48 h, every 8 h for the next 24 h, and every 12 h for the next 48 h.

Dry matter content of feed and faeces were determined by drying samples at 60°C in a fan-forced oven to a constant weight. Samples were ground through a 1 mm sieve before analysis of Cr and Co concentrations (Barnett *et al.* 2016) using a portable X-ray fluorescence spectrometer (Bruker Tracer III-V pXRF, Bruker Corp, Billerica, MA USA). Analysis of digesta kinetics was undertaken using non-linear curve fitting algorithms of WinSAAM (Aharoni *et al.* 1999).

The concentration of allantoin in the urine was determined by the colourimetric method (IAEA 1997), using a UV-1201 spectrophotometer (Shimadzu, Japan) reading at 522nm. The yield of total microbial crude protein (MCP) from the rumen was calculated from allantoin excretion using equations of Chen *et al.* (1992).

4.2.7 Rumen fluid sampling, ammonia, volatile fatty acid concentrations, and protozoal enumeration

Samples of rumen fluid (20 mL) were collected from each lamb before feeding using oesophageal intubation for protozoal enumeration, volatile fatty acid (VFA) and ammonia (NH_3) analyses on days 0, 25, 39, 65 and 92. Rumen pH was measured immediately using a portable pH meter (Orion 230 Aplus, Thermo scientific, Beverly, MA, USA). A 15 mL subsample was placed in wide-neck McCartney bottle acidified with 0.25 mL of 18 M sulphuric acid and stored at -20^{0}C for VFA and NH_3 analyses. A 4 mL subsample was placed in wide-neck McCartney bottle containing 16 mL of formaldehyde-saline (4% formalin v/v) for protozoa enumeration. Protozoa were counted using a Fuchs–Rosenthal optic counting chamber (0.0625 mm2 and 0.2 mm of depth) using a staining technique adapted from the procedure described by Dehority (1984). The protozoa were differentiated into large (>100 µm) and small (<100 µm) holotrichs and entodiniomorphs. The VFA concentrations were determined by gas chromatograph (Nolan *et al.* 2010) using a Varian CP 3800 Gas Chromatograph (Varian Inc. Palo Alto, California USA) and NH_3 concentration was analysed using a modified Berthelot reaction using a continuous flow analyser (San++, Skalar, Breda, The Netherlands).

4.2.8 Liveweight and clean wool growth

Lambs were weighed in the morning prior to feeding on days 0, 15, 21, 30, 65 and 93 to monitor liveweight and determine average daily gain (ADG) over the experiment. Clean wool growth (CWG) rate, wool yield, and fibre diameter were determined on the mid-

side of the sheep from day 25 to day 92 by clipping a patch 10×10 cm (Oster Golden A5 clippers, blade size 30 model, Cryogen X, USA). After the wool from the patch was clipped, four sides and one diagonal were measured and the area of the patch was calculated using Heron's formula (De Barbieri *et al.* 2014). Wool samples were sent to New England Fibre Testing Pty Limited to determine yield and fibre diameter.

4.2.9 Statistical analyses

Data was statistically analysed using SAS 9.0 (SAS Institute, Cary, NC). Data for rumen fermentation characteristics, digesta kinetics, CWG, MCP outflow and DMP were subject to analysis of variance in PROC GLM; factors being protozoa, NO_3 and protozoa × NO_3 interaction. For analysis of final liveweight and ADG, the model used the initial liveweight as a covariate. For parameters which had more than one measures, all measures were averaged. Homogeneity of variance and normal distribution were tested using PROC UNIVARIATE before statistical analysis. Data on MetHb and protozoa count were log-transformed before statistical analysis. For all analyses, means were analysed using the least squares means (LSMEANS) procedure and a probability of < 5% was considered to be statistically significant.

4.3 Results

4.3.1 Blood methaemoglobin concentration

The averaged, blood MetHb concentration over the whole experiment was significantly increased by defaunation (P = 0.03) and by supplementation of NO_3 (P < 0.01) and there was a significant interaction between defaunation and NO_3 supplementation on

MetHb (P = 0.04; Table 4.2). In defaunated lambs on NO_3, two lambs were observed having MetHb values of 18.1 and 18.3% on day 50 and one lamb observed with MetHb value of 19.1% on day 85. In faunated lambs on NO_3, the highest MetHb was one lamb found with 6.2% of MetHb on day 50.

Table 4.2 Rumen fermentation characteristics, concentration of methaemaglobin (MetHb) and protozoal population of defaunated (-P) and faunated lambs (+P) fed diets of oaten chaff with or without nitrate (NO_3) supplementation.

Parameter	Treatment				Pooled s.e.	P-Values		
	-P		+P			P	NO_3	P × NO_3
	- NO_3 (n = 5)	+ NO_3 (n = 5)	- NO_3 (n = 5)	+ NO_3 (n = 5)				
Rumen pH	6.72	6.61	6.69	6.57	0.03	0.16	<.001	0.98
Total VFA (mM/L)	32.71	37.71	35.17	49.03	3.10	0.04	0.01	0.17
Acetate (mol %)	72.89	72.62	68.15	71.02	0.95	<.01	0.18	0.11
Propionate (mol %)	17.15	15.42	20.63	17.79	1.18	0.02	0.06	0.63
Butyrate (mol %)	8.00	9.58	9.51	9.42	0.75	0.38	0.34	0.28
Acetate : propionate ratio	4.37	4.76	3.34	4.07	0.30	0.01	0.08	0.59
NH_3-N (mg/L)	6.04	21.51	11.21	30.70	2.72	0.02	<.001	0.46
MetHb (%)	0.91	5.48	0.87	1.55	0.94	0.03	<0.01	0.04
Total protozoa ($\times 10^5$/mL)	0	0	4.78	6.51	0.61		0.20	
Small entodiniomorph	0	0	4.08	5.54	0.53		0.19	
Large entodiniomorph	0	0	0.18	0.13	0.08		0.51	
Small holotrich	0	0	0.50	0.79	0.19		0.33	
Large holotrich	0	0	0.02	0.05	0.02		0.42	

4.3.2 Rumen fermentation and methane emissions

Defaunated lambs remained protozoa-free throughout the experiment. In faunated lambs, NO_3 supplementation did not affect protozoal population ($P > 0.05$; Table 4.2). Small entodiniomorphs in both NO_3 and non-nitrate supplemented lambs accounted for 85% of total protozoa.

Nitrate supplementation significantly increased concentration of NH_3-N and total VFA ($P < 0.05$; Table 4.2). There was a tendency towards a lower molar percentage of propionate ($P = 0.06$) and higher molar ratio of acetate to propionate ($P = 0.08$) in nitrate-supplemented lambs. Defaunation, contrastingly, decreased total VFA, NH_3-N concentration and molar percentage of propionate. Defaunation increased the molar percentage of acetate and molar ratio of acetate to propionate ($P < 0.05$). Rumen pH was not affected by defaunation, but was decreased by NO_3 supplementation (Table 4.2). No interactions between defaunation and NO_3 were found for any rumen fermentation parameter.

Daily methane production was significantly decreased by defaunation, but was increased by NO_3 supplementation ($P < 0.001$; Table 4.3) with no interaction between defaunation and NO_3 for DMP. There was a positive correlation between DMI and DMP (DMP = 0.014 DMI - 3.38; $r^2 = 0.75$, $P = 0.001$), such that DMP was significantly increased by higher DMI. Defaunation significantly decreased CH_4 yield (MY, g CH_4/kg DMI) but NO_3 did not change MY. However, there was a significant interaction between defaunation and NO_3 ($P = 0.01$) in MY such that in defaunated lambs, NO_3 did not change MY, but in faunated lambs, NO_3 decreased MY by 25%. Nitrate-

supplemented defaunated lambs had lower MY than nitrate-supplemented faunated lambs ($P < 0.001$; 10.14 *v.* 14.20 g/kg DMI).

4.3.3 Performances and digestion

Productivity of lambs was significantly increased by NO_3 supplementation, but was not affected by defaunation. Supplementation of oaten chaff with NO_3 significantly increased DMI from 622 to 895 g/d and DMD from 57.8 to 64.5% ($P < 0.001$; Table 4.3). Nitrate supplementation significantly increased MCP outflow, ADG, CWG and wool fibre diameter ($P < 0.01$). The intakes of ME and CP were significantly increased by NO_3 supplementation from 3.8 to 6.1 MJ/d and from 14.9 to 41.7 g/d, respectively. Defaunation did not change DMI, ADG, MCP outflow or CWG, but decreased DMD ($P = 0.05$). There were no significant interactions between defaunation and NO_3 supplementation for productivity parameters.

Digesta kinetics as characterised by MRT of both rumen solute and particulate fractions was significantly affected by both defaunation and NO_3 supplementation. Defaunation significantly increased rumen MRT of solute and particulate fractions ($P < 0.05$) while NO_3 supplementation significantly decreased rumen MRT of these fractions ($P < 0.05$). There was a negative correlation between MRT and DMI across all data (particulate MRT = 70.8 - 0.028 DMI, $r^2 = -0.52$, $P = 0.038$ and solute MRT = 52.2 - 0.028 DMI, $r^2 = -0.67$, $P = 0.004$), such that greater DMI was associated with shorter marker MRT in the rumen. Despite the slowing effect of defaunation and accelerating effect of NO_3 on rumen MRT, there were no significant effects of defaunation or NO_3 on MRT of solute

and particulate fractions in the hindgut. There were no interactions between defaunation and NO_3 in other gut segment, except hindgut particulate MRT ($P = 0.02$).

Table 4.3 Intake, productivity, methane emissions and digesta kinetics of defaunated (-P) and faunated lambs (+P) fed diets of low-protein oaten chaff with or without nitrate (NO_3) supplementation.

Parameter	Treatment				Pooled s.e.	*P*-Values		
	-P		+P			P	NO_3	P × NO_3
	- NO_3 (n = 5)	+ NO_3 (n = 5)	- NO_3 (n= 5)	+ NO_3 (n= 5)				
DMI (g/d)	620.71	849.32	624.61	941.72	53.79	0.37	<.001	0.41
ME intake (MJ/d)	3.61	5.75	4.08	6.48	0.45	0.20	<.001	0.77
CP intake (g/d)	13.97	39.23	15.77	44.21	2.75	0.24	<.001	0.57
Final LW(kg)	35.42	40.31	32.62	39.84	1.30	0.14	<.001	0.37
ADG (g/d)	-0.95	54.55	-32.81	49.26	14.77	0.21	<.001	0.39
CWG (µg/cm^2.d)	468	662	482	704	53.5	0.61	<.01	0.79
Wool fibre diameter (µm)	18.85	22.87	19.58	21.96	0.82	0.96	<.01	0.35
DMP (g CH_4/d)	3.49	7.65	7.13	12.29	0.85	<.001	<.001	0.57
MY (g CH_4/kg DMI)*	7.30	10.14	18.88	14.20	1.31	<.001	0.50	0.01
DMD (%)	54.88	63.77	60.81	65.25	1.72	0.05	<.01	0.22
MCP outflow (g/d)	3.17	8.55	3.14	8.29	1.45	0.89	<.01	0.96
Rumen particulate MRT(h)	40.20	32.83	29.10	21.68	1.74	<.001	<.01	0.98
Hindgut particulate MRT(h)	15.05	20.53	21.97	14.70	2.33	0.82	0.71	0.02
Total particulate MRT(h)	55.25	53.35	50.82	36.37	3.66	0.01	0.05	0.11
Rumen solute MRT(h)	25.37	19.20	20.75	14.10	1.90	0.03	0.01	0.90
Hindgut solute MRT(h)	12.25	12.65	15.20	10.28	1.52	0.85	0.16	0.11
Total solute MRT(h)	37.63	31.85	35.95	24.38	3.22	0.18	0.02	0.38

*(n = 4) during measures of methane emissions and total collection; Dry matter intake (DMI); Average daily gain (ADG); Clean wool growth (CWG); Daily methane production (DMP); Methane yield (DMD); Dry matter digestibility (DMD); Microbial crude protein (MCP); Mean retention time (MRT); *DMI was calculated during restricted intake period.*

4.4 Discussion

4.4.1 Effects of nitrate on blood MetHb concentration and protozoa population

Nitrate toxicity remains a major constraint to commercial NO_3 feeding because excessive NO_3 in the rumen may accumulate nitrite (NO_2) concentrations in the rumen and then blood. Nitrite in blood reduces the ferric ion of haemoglobin and transforms the molecule to MetHb (Lundberg *et al.* 2008), which is unable to transport oxygen to tissues. Methaemoglobinaemia is diagnosed if more than 30% of haemoglobin is present on MetHb (Bruning-Fann and Kaneene 1993). In this study, feeding faunated lambs with 3.1% calcium NO_3 (~2% NO_3) maintained low MetHb concentration in agreement with previous studies when NO_3 was supplemented at levels up to 2.6% by gradually introducing NO_3 to allow adaptation of rumen microbes (Nolan *et al.* 2010; van Zijderveld *et al.* 2010; Li *et al.* 2012). This is because rumen microbes are capable of reducing NO_3 or NO_2 to NH_3 as NO_3 is introduced as reviewed by Leng and Preston (2010).

Defaunated lambs in the present study showed increased MetHb concentration after 85 days of feeding NO_3, suggesting that protozoa may have an important role in the reduction of NO_3 or NO_2 in the rumen and consequently formation of MetHb in the blood of sheep. Lin *et al.* (2011) incubated different microbial fractions of whole rumen fluid, protozoa, bacteria, and fungi to assess their ability to reduce NO_3. The authors found that NO_3 disappearance rate was similar in whole rumen fluid and protozoal fractions. Nakamura and Yoshida (1991) also reported that NO_3 and NO_2 disappearance

rates in the rumen of faunated sheep were faster than in defaunated sheep and lower MetHb was observed in faunated sheep, potentially indicating active involvement of protozoa in the reduction of NO_3 and NO_2. However, there were no clinical signs of NO_3 toxicity in either defaunated and faunated lambs when 2% NO_3 was supplemented in these *ad libitum* fed lambs.

The protozoal population was not affected by NO_3 supplementation in this study; this agreed with previous studies (Nolan *et al.* 2010; van Zijderveld *et al.* 2010; Li *et al.* 2012).

4.4.2 Effects of nitrate supplementation and defaunation on performances and digestion

Oaten chaff used in this study as a basal diet for lambs was characterized by a low protein content (41 g CP per kg DM) providing only 4 g of CP per MJ of ME. Consequently, this diet was inadequate to support maintenance of growing lambs (CSIRO 2007), resulting in losing weight in lambs without NO_3 supplementation. Similarly, the average value of 7 g of CP per MJ of ME in the nitrate-supplemented diet was below that required to support rumen fermentation required for growing sheep (CSIRO 2007). The averaged concentrations of NH_3-N in the rumen increased from 8.6 to 26 mg/L rumen fluid with NO_3 and were associated with increased MCP outflow (3.2 to 8.4 g/d). However, it was suggested by Satter and Slyter (1974) that 20-50 mg NH_3-N/L rumen fluid is required to maintain growth of rumen bacteria with forage diets, so the amount of NH_3-N even in nitrate-supplemented lambs in this study was sub-optimal, although it still increased microbial growth and activity as indicated by

greater total VFA compared to unsupplemented lambs. Lambs supplemented with NO_3 had an ADG of 52 g/d and grew 683 µg/cm^2.d of CWG, suggesting high efficiency of nutrient utilisation by lambs on NO_3 supplementation. Lambs without NO_3 lost 25 g LW/d and CWG grew 475 µg/cm^2.d, suggesting that wool growth was utilising amino acids as a priority over body growth.

Defaunation resulted in a significant reduction of rumen NH_3-N concentration as less digestion of engulfed feed-protein and bacteria occurs in the absence of protozoa; this agreed with previous assessments (Jouany *et al.* 1988; Eugène *et al.* 2004a; Santra *et al.* 2007a; Morgavi *et al.* 2012). Defaunated lambs in this study had 13.8 mg NH_3-N/L rumen fluid, which was below the requirement for the maximum growth of microbes (Satter and Slyter 1974) and thus inadequate NH_3-N availability inhibited ruminal fermentation (Leng 1990). This resulted in lower total VFA, DMD and longer MRT, but no changes in MCP outflow, ADG or CWG by defaunated lambs. This contrasts with previous results where defaunation increased rumen bacterial outflow and increased the availability of protein at the duodenum (Bird and Leng 1978; Jouany 1996).

The 30% increase in DMI by nitrate-supplemented lambs was probably due to increased NH_3-N and fermentation. The negative correlation between DMI and particulate and soluble MRT (r^2= -0.52 and -0.67; $P < 0.05$) may reflect the positive role of NH_3 in stimulating feed breakdown in the rumen and enabling additional feed intake. The shorter MRT in NO_3 fed lambs allowed these animals to consume more feed due to a reduced rumen fill constraint, faster passage and greater fermentation. In addition, greater whole tract DMD would have increased CP and ME intake supporting the

suggestion of Leng (1990) that harvesting of nutrients from low-quality forages can be improved by ruminants if microbes in the rumen grow efficiently. This is in keeping with the finding that lambs supplemented with NO_3 in this study had higher ADG and CWG than unsupplemented lambs. In contrast, NO_3 did not increase DMI, DMD and ADG in previous studies where protein was above ruminal requirement (van Zijderveld *et al.* 2010; Li *et al.* 2012; de Raphélis-Soissan *et al.* 2014). However, because those studies aimed to replace urea to NO_3 in nitrogen-adequate diets, the authors were unlikely to observe positive effects of NO_3 on fermentation and productivity of animals as reported here in the protein deficient diet.

4.4.3 Effects of nitrate supplementation and defaunation on methane emissions and rumen fermentation

The higher DMP in nitrate-supplemented lambs contrasts with results from protein adequate diets and was a consequence of higher DMI and increased ruminal fermentation as evidenced by higher total VFA concentration and DMD, leading to greater ruminal H_2 availability. In faunated lambs, the 24.8% reduction in MY by NO_3 supplementation agrees with previous studies which have shown CH_4 mitigation ranges between 23 and 35% when 1.9 to 2.6% NO_3 were supplemented (Nolan *et al.* 2010; van Zijderveld *et al.* 2010; Li *et al.* 2012). A review by Leng and Preston (2010) showed that CH_4 can be reduced by 16 to 50% depending on diets and the inclusion rate of NO_3. As the same amount of H_2 is used to reduce 1 mol of NO_3 to NH_3 as 1 mol of CO_2 to CH_4 (Nolan *et al.* 2010), the faunated lambs in this study were given 2% NO_3 (14.3 g NO_3 per day during the restricted intake period), which theoretically could reduce 0.23

mol or 4.92 g CH_4/kg DMI. In this study, a reduction of 4.68 g CH_4/kg DMI was measured, which is 95% of the expected reduction, showing that most of the calcium NO_3 was reduced to NH_3-N. Nitrate caused changes in rumen fermentation shifting to increased acetate and decreased propionate as high affinity H_2 of NO_3 is more favourable in NO_3 reduction than in formation of propionate or CH_4 (Ungerfeld and Kohn 2006). The present study showed a tendency of lower propionate and higher molar ratio of acetate to propionate, which was consistent with previous observations by Nolan *et al.* (2010). The reduced CH_4 production by NO_3 supplementation may also be a consequence of inhibiting methanogens (van Zijderveld *et al.* 2010) as the reduction of NO_3 to NO_2 and then to NH_3 resulted in a metabolic H_2 sink, which decreased H_2 availability for methanogens (van Zijderveld *et al.* 2011).

The reduced DMP after defaunation is consistent with Hegarty (1999) and can be explained by fermentation shifting to a greater proportion of propionate and decreasing the proportion of butyrate. However, results from this study showed DMP was reduced with decreased total VFA and propionate proportion, but an increased acetate proportion. A higher proportion of acetate and lower proportion of propionate in defaunated animals fed low quality diets was also reported by Bird (1982). The lower DMP in defaunated lambs could be due to restricted growth of microbes in the rumen, evidenced by the lower fermentation, NH_3 concentration and DMD of defaunated lambs. Alternatively, by removing the endo-symbiotic and ecto-symbiotic methanogens associated with protozoa, H_2 may have accumulated, stimulating reductive acetogenesis (Ungerfeld 2013). Fonty *et al.* (2007) also reported that reductive acetogens established in the rumen lacking methanogens and can replace methanogens as a sink for H_2 in the

rumen, thus reductive acetogens can be potentially important to reduce enteric CH_4 emissions (Joblin 1999). Because reductive acetogenesis involves the reduction of CO_2 by H_2 to acetate (Ungerfeld 2013), this might explain the low CH_4 emissions, but high acetate concentration in defaunated lambs. However, as H_2 concentration and methanogen population were not measured in this study, it is not possible to confirm that hypothesis.

4.4.4 Interaction of defaunation and nitrate supplementation

In the present study, significant interactions of protozoa and NO_3 occurred for MetHb and MY. Concerning the role of protozoa on NO_3/NO_2 reduction and MetHb, the present study confirmed the *in vitro* study by Lin *et al.* (2011) and showed that blood MetHb became greater in defaunated lambs after 85 days of feeding NO_3, which was also in agreement with a study by Nakamura and Yoshida (1991) who reported that NO_3 disappearance rates and blood MetHb were rapidly decreased in faunated animals compared to defaunated animals. In feeding NO_3 to defaunated and faunated lambs in this study it was hypothesised that dietary NO_3 in combination with defaunation treatment would additively decrease methanogens and DMP. Methanogens use H_2 as a substrate for their metabolism and the use of H_2 by bacteria to reduce NO_3 to NO_2 and then NH_3 can cause lower numbers of methanogens (van Zijderveld *et al.* 2010; van Zijderveld *et al.* 2011). The combined treatment of NO_3 and defaunation could be expected to cause greater effects on reducing methanogen population. A significant interaction between protozoa and NO_3 supplementation on MY occurred suggesting that

NO_3 supplemented to defaunated lambs would be positively additive in lowering MY compared to faunated lambs with or without NO_3 supplementation.

4.5 Conclusion

Nitrate is an effective NPN source for rumen microbes, especially in a protein deficient diet. From the point view of greenhouse gas mitigation, NO_3 is an effective strategy to reduced enteric CH_4 emissions, provided it is supplemented at appropriate levels. Defaunation reduced fermentation, DMP and digestion with no changes in MCP outflow or ADG. Moreover, fermentation and digestion of defaunated lambs were increased by NO_3 supplementation and the combined treatments of defaunation and NO_3 were additive in reducing CH_4 yield. This needs further investigation as combining two CH_4 mitigation strategies may be an effective approach in delivering significant methane mitigation by grazing livestock.

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Chapter 5

Effects of rumen protozoa of Brahman heifers and nitrate on fermentation and *in vitro* methane production

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Abstract

Two experiments were conducted assessing the effects of presence or absence of rumen protozoa and dietary nitrate addition on rumen fermentation characteristics and methane (CH_4) production in Brahman heifers. The first experiment assessed changes in rumen fermentation pattern and *in vitro* CH_4 production post-refaunation and the second experiment investigated whether addition of nitrate to the incubation would give rise to CH_4 mitigation additional to that contributed by defaunation. Ten Brahman heifers were progressively adapted to a diet containing 4.5% coconut oil distillate (COD) for 18 days and then all heifers were defaunated using sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol). After 15 days, the heifers were given a second dose of Empicol. Fifteen days after the second dosing, all heifers were allocated to defaunated or refaunated groups by stratified randomisation based on liveweight, and the experiment commenced (day 0). On day 0, an oral dose of rumen fluid collected from unrelated faunated cattle was used to inoculate 5 heifers and form a refaunated group so that the effects of re-establishment of protozoa on fermentation characteristics could be investigated. Samples of rumen fluid collected from each animal using oesophageal intubation before feeding on days 0, 7, 14 and 21 were incubated for *in vitro* CH_4 production. On day 35, 2% of nitrate (as $NaNO_3$) was included in *in vitro* incubations to test for additivity of nitrate and of protozoa effects on fermentation and CH_4 production. It was concluded that increasing protozoal numbers were associated with increased CH_4 production in refaunated heifers 7, 14 and 21 days after refaunation. Methane production rate was significantly higher from refaunated heifers than from defaunated heifers 35 days after refaunation. Concentration and proportions of major volatile fatty acids, however, were not affected

by protozoal treatments. There is scope for further reducing CH_4 output through combining defaunation and dietary nitrate as the addition of nitrate in the defaunated heifers resulted in 86% reduction in CH_4 production *in vitro*.

Key words: Defaunation, refaunation, nitrate, methane production.

5.1 Introduction

Reviews of the effects of enteric protozoa on digestion and productivity by ruminants have concluded removal of rumen ciliate protozoa reduces enteric methane (CH_4) emissions by 11% (Newbold *et al.* 2015) and increases an average daily gain by 11% (Eugène *et al.* 2004a). Finlay *et al.* (1994) concluded that methanogens existing as endo- and ecto-symbionts with ciliate protozoa contributed 37% of rumen CH_4 production and Stumm *et al.* (1982) identified that 10 to 20% of rumen methanogens were attached on the outside of protozoa. Centrifuging rumen fluid to remove protozoa reduced the methanogen population by 78% (Newbold *et al.* 1995).

Methane production is positively related to the size of the rumen protozoal population (Morgavi *et al.* 2010) and the absence of protozoa reduces CH_4 production and significantly modifies fermentation characteristics *in-vitro* (Qin *et al.* 2012). However, Ranilla *et al.* (2007) reported that there was no correlation between methanogenesis and protozoal biomass per unit of feed degraded *in-vitro*. Further, Bird *et al.* (2008) showed that defaunation did not change enteric CH_4 production 10 to 25 weeks post-treatment. Hegarty *et al.* (2008) also reported that rumen protozoa did not affect CH_4 production by lambs raised without protozoa from birth, or defaunated at weaning. Therefore, the relationship between rumen protozoa and enteric CH_4 production is unclear.

In contrast, dietary nitrate reduces CH_4 production reliably and predictably (van Zijderveld *et al.* 2010; van Zijderveld *et al.* 2011). Nitrate reduces total gas production when rumen fluid is incubated *in vitro*, and it changes the volatile fatty acid (VFA) profile by increasing acetate and reducing propionate and butyrate molar proportions while total volatile fatty acid concentration is unaffected (Lin *et al.* 2011). The objectives of these studies were to describe the fermentation characteristics and CH_4 production changes occurring in the period after refaunation of previously protozoa-free heifers, and assess whether nitrate could further reduce CH_4 production from defaunated animals.

5.2 Materials and methods

5.2.1 Animals and feeding

All protocols for treatment and care of the cattle were approved by the University of New England Animal Ethics Committee (AEC 13-054). Ten Brahman heifers (8 months of age) with an average liveweight of 274 ± 32.8 kg were used. Cattle were adapted to a pre-experimental diet of oaten (70%) and lucerne (30%) chaff with initial inclusion of 1% of coconut oil distillate (COD) which was raised to a final level of 4.5% over 8 days. Cattle were then changed to an experimental diet for 10 days to eliminate rumen protozoa comprising oaten chaff (70%), lucerne chaff (21%), COD (4.5%) and molasses (4.5%), resulting in 88.1% DM in the mixed ration and 7.9 % crude protein and 5% crude fat in the dry matter. This combined 18 day period of COD dietary treatment reduced the protozoal population from 3.91×10^5 cells/mL to 0.58×10^5 cells/mL of rumen fluid and all cattle were then treated with a chemical to defaunate. After the

defaunation treatment, all cattle were given a diet of oaten (70%) and lucerne chaff (30%) which included 10.5% crude protein; 1.3 crude fat; 88.8% dry matter for the remainder of the study. All cattle had *ad libitum* access to the ration and water.

5.2.2 Defaunation of cattle

After 18 days feeding COD, all feed was withdrawn for a day and cattle were orally dosed with sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol ESB/70AV, Albright and Wilson Australia Ltd, Melbourne) administered at 45g/d in a 10% v/v solution to remove protozoa. Cattle were dosed on three consecutive days and feed was withheld during this treatment protocol, which was based on that of Bird and Light (2013). Animals required 15 days to fully recover their previous voluntary intake and received the COD diet during this period of time. The three day dosing with Empicol was then repeated commencing 15 days after the first dosing. A further 15 days after the second drenching program, rumen fluid samples were collected for protozoa enumeration and the experiment commenced (d 0).

5.2.3 Refaunation of cattle

On day 0 all cattle had recovered their intake and wellbeing, and rumen fluid of the animals was observed to be free of protozoa. Cattle were allocated to defaunated (n=5) and refaunated groups (n=5) by stratified randomisation based on liveweight. A single oral dose (500 mL/heifer) of a mixed rumen fluid collected from two cannulated faunated cattle grazing pasture was used to refaunate 5 heifers. The protozoal population in the inoculum (3.42×10^5 cells/mL) consisted of large holotrichs (0.13×10^5

cells/mL), small holotrichs (0.5×10^5 cells/mL) and small entodiniomorphs (2.79×10^5 cells/mL).

5.2.4 Rumen fluid sampling, ammonia, volatile fatty acid concentrations, and protozoal enumeration

In Experiment 1, samples of rumen fluid (40 mL) were collected using oesophageal intubation from defaunated and refaunated heifers before feeding on days 0, 7, 14 and 21. Samples from defaunated heifers were immediately checked under a microscope to confirm that defaunated heifers were protozoa-free. Rumen pH was measured immediately using a portable pH meter (Orion 230 Aplus, Thermo scientific, Beverly, MA, USA). A 15 mL subsample was placed in wide-neck McCartney bottle acidified with 0.25 mL of 18 M sulphuric acid and stored at -20^0C for VFA and ammonia (NH_3) analyses. A 4 mL subsample was placed in wide-neck McCartney bottle containing 16 mL of formaldehyde-saline (4% formalin v/v) for protozoa enumeration. Protozoa were counted using a Fuchs–Rosenthal optic counting chamber (0.0625 mm2 and 0.2 mm of depth) using a staining technique adapted from the procedure described by Dehority (1984). The protozoa were differentiated into large (>100 µm) and small (<100 µm) holotrichs and entodiniomorphs. Another 20 mL of subsample from defaunated and refaunated heifers was used to conduct *in vitro* incubations for CH_4 measurement.

In Experiment 2, samples of rumen fluid (~20 mL) were collected on day 35 using oesophageal intubation from defaunated and refaunated heifers before feeding with each sample being processed individually and its incubation started immediately after collection.

Concentration of VFA were determined (Nolan *et al.* 2010) using a Varian CP 3800 Gas Chromatograph (Varian Inc. Palo Alto, California USA) and NH_3 concentration was analysed using a modified Berthelot reaction using a continuous flow analyser (San^{++}, Skalar, Breda, The Netherlands).

5.2.5 *In vitro* incubations and measurements

In vitro incubations (23h) were conducted using rumen fluid collected from defaunated and refaunated heifers on days 0, 7, 14 and 21 after refaunation, to assess changes in CH_4 production in defaunated heifers and refaunated heifers while rumen protozoa were re-establishing in refaunated heifers (Experiment 1). Samples were then taken on day 35 and incubated *in vitro* with the addition of 2% nitrate (NO_3 as $NaNO_3$) to test for additivity of NO_3 and defaunation effects on fermentation and CH_4 production (Experiment 2). The $NaNO_3$ was dissolved in purified water and added in buffer solution. The composition of incubation buffer was adapted and modified after (Soliva and Hess 2007). For all *in vitro* incubations, 20 mL of rumen fluid from each animal was injected into a Schott bottle (100 mL) which contained 40 mL of buffer solution under a constant flow of anaerobic CO_2 in a water bath maintained at 39^0C. Mixed rumen fluid and buffer solution (10mL) was transferred into three 50 mL syringes (Luer lock: Terumo Corporation, Japan) which contained 200 ± 20 mg of ground substrate (70% oaten and 30% lucerne chaff). The syringes were sealed by a 3-way tap, pre-warmed to 39^0 C and then incubated in a shaking water bath at 39^0 C. After the incubations, gas volume was measured, liquid was drained from the syringes and placed in wide-neck McCartney bottle acidified with 0.25 mL of 18 M sulphuric acid and

stored at -20^{0}C for VFA and NH_3 analyses. The gas in the syringes were analysed for CH_4 concentration using a gas chromatograph (SMARTGAS, Varian CP 4900).

5.2.6 Statistical analyses

Data were statistically analysed using SAS 9.0 (SAS Institute, Cary, NC). Data from Experiment 1 were subject to repeated-measures analysis of variance with protozoa, time and protozoa × time interaction as fixed factors. Data from Experiment 2 were subject to analysis of variance in PROC GLM, factors being protozoa, NO_3 and protozoa × NO_3 interaction. Means were analysed using the least squares means (LSMEANS) procedure. A probability of < 5% was considered to be statistically significant.

5.3 Results

5.3.1 Protozoal population in refaunated heifers

Protozoa were not observed in any rumen fluid samples collected from defaunated heifers during this study. In refaunated heifers, however, the protozoal population reached 3.70×10^5 cells/mL by day 7 and almost doubled by day 21 (7.01×10^5 cells/mL). Small entodiniomorphs were predominant in the total population, accounting for 94, 82 and 86 % of the total counts at days 7, 14 and 21, respectively (Figure 5.1). Methane production from refaunated cattle was positively correlated with protozoal numbers although CH_4 production tended to stabilise after day 14 (Figure 5.2).

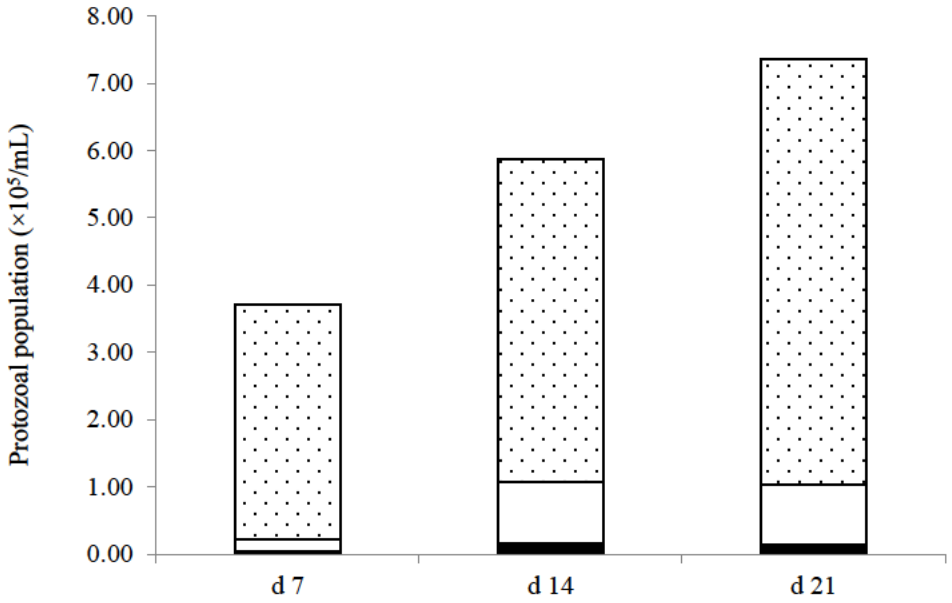

Figure 5.1 Small Isotrichs (□), large Isotrichs (■) and small Entodiniomorphs ▒ from refaunated heifers 7, 14 and 21 days after refaunation.

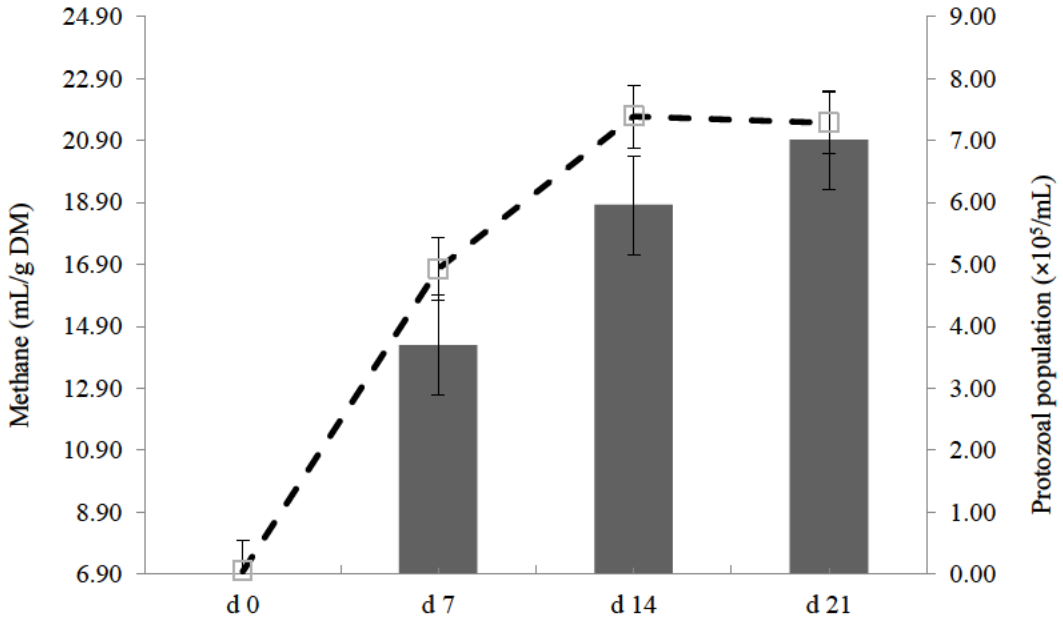

Figure 5.2 Methane production (□) and protozoal numbers (■) in rumen fluid from refaunated heifers 0, 7, 14 and 21 days after refaunation using a mixed rumen fluid inoculum. Error bars indicate s.e.m.

5.3.2 Fermentation pattern and methane production in Experiment 1

The rumen fluid pH was higher in refaunated heifers, but increased from day 0 to day 21 in both defaunated and refaunated heifers, showing effects of protozoal treatments and time (Table 5.1). Ammonia concentrations increased steadily up to day 7 in both defaunated and refaunated heifers, but refaunated heifers had higher NH_3 concentrations than did defaunated heifers ($P < 0.05$). Neither VFA concentration, nor molar proportions of acetate, propionate and butyrate in total VFA, or acetate to propionate ratio were affected by protozoal treatment, but all except butyrate proportion increased over time.

There was an increase in total gas production *in vitro* by rumen fluid collected from both defaunated and refaunated heifers from day 0 to day 14 with no significant further increase to day 21. There was a tendency towards a lower CH_4 production from rumen fluid of defaunated heifers than from refaunated heifers over time ($P = 0.07$). No significant interaction between protozoal treatment and time were observed ($P > 0.05$).

Table 5.1 The pH, ammonia concentration and concentration and molar proportions of major volatile fatty acids (VFA) in rumen fluid, and changes in gas and methane production *in vitro* after refaunation.

Item[a]	Treatment								s.e.m	*P*-values		
	-P (n = 6)				+P (n = 6)					P	Day	P × Day effect
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21				
pH	6.41	6.46	6.87	6.83	6.62	6.69	6.86	6.91	0.10	0.02	<0.001	0.34
NH_3-N (mg/L)	32.68	30.76	59.04	62.92	36.88	69.52	86.24	117.00	9.56	<0.01	<0.001	0.08
Total VFA(mM/L)	64.43	59.67	50.92	57.95	59.46	63.43	63.03	58.16	8.05	0.63	0.18	0.39
Acetate (molar %)	71.06	74.55	75.32	79.01	73.67	73.49	73.39	76.74	1.76	0.59	0.04	0.51
Propionate (molar %)	19.15	16.61	15.05	14.46	17.75	15.66	14.52	12.30	1.40	0.12	0.02	0.95
Butyrate (molar %)	8.38	7.05	6.54	6.39	6.77	8.03	8.44	7.39	0.65	0.37	0.60	0.03
Acetate /propionate	4.07	4.65	5.08	5.57	4.58	4.77	5.07	6.29	0.51	0.26	0.44	0.90
Total gas[b] (mL/g DM)	102.33	128.67	144.07	157.00	103.67	135.67	152.00	149.33	4.71	0.55	<0.001	0.34
CH_4 (mL/g DM)	6.44	13.60	16.86	20.66	6.99	16.76	21.68	21.47	1.29	0.07	<0.001	0.19

-P (defaunated), +P (refaunated); Standard error of the mean (s.e.m); [a] pH, ammonia and VFA analyses on samples collected from animals on days 0, 7, 14 and 21; [b] Gas and methane production data collected from in-vitro incubations.

5.3.3 Fermentation pattern and methane production in Experiment 2

The pH after incubation was increased by the presence of protozoa and by NO_3 ($P < 0.05$; Table 5.2). Ammonia concentration was also increased by the presence of protozoa and by NO_3 ($P < 0.05$). The presence of protozoa had little effect on VFA, with total VFA concentration tending to be lower in rumen fluid from defaunated than refaunated heifers, but VFA proportions were unaffected. Total VFA concentration was significantly reduced by NO_3 and a significant reduction in butyrate percentage also occurred.

Table 5.2 The pH, ammonia concentration, volatile fatty acid concentration and molar proportions and methane production as influenced by the presence or absence of protozoa or nitrate addition in incubations of rumen fluid *in vitro.*

Parameter	Treatment				s.e.m	*P*-Values		
	-P (n =6)		+P (n =6)			P	NO_3	P × NO_3
	-NO_3	+NO_3	-NO_3	+NO_3				
pH	6.19	6.49	6.02	6.32	0.05	<.01	<.01	0.98
NH_3-N (mg/L)	101.19	185.71	167.23	211.70	11.60	0.01	0.01	0.18
Total VFA (mM/L)	102.96	83.15	137.57	98.01	12.14	0.08	0.04	0.14
Acetate (molar %)	69.33	70.30	67.75	68.74	1.95	0.45	0.63	0.10
Propionate (molar %)	20.34	22.48	19.57	21.54	1.14	0.47	0.11	0.94
Butyrate (molar %)	9.57	6.78	10.96	8.49	1.10	0.20	0.04	0.89
Acetate/propionate	3.42	3.15	3.47	3.26	0.26	0.76	0.39	0.92
Total gas (mL/g DM)	155.00	101.67	149.44	117.78	4.14	0.21	<.01	0.01
CH_4 (mL/g DM)	18.59	3.00	22.11	12.73	0.63	<.01	<.01	<.01

Standard error of the mean (s.e.m); -P (defaunated), +P (refaunated).

Methane production was reduced by both defaunation and by NO_3, and there was a significant interaction between defaunation and NO_3 such that mitigation resulting from NO_3 and defaunation was greater than the mitigation resulting from either alone ($P < 0.05$). Methane production from defaunated heifers was lower than from refaunated heifers (18.59 v 22.11 mL/g DM). While NO_3 reduced CH_4 production in refaunated heifers (12.73 v 22.11 mL/g DM), the combined effects of defaunation and dietary NO_3 on CH_4 mitigation (19.11 mL) was greater than the sum of effects of defaunation (3.52 mL) and NO_3 (9.38 mL) alone, implying the combined treatments were synergistic in their mitigation potential. Total gas production was not affected by protozoal treatments ($P > 0.05$), but was reduced in incubations containing NO_3 ($P < 0.05$).

5.4 Discussion

The objectives of this study were to describe the changes in CH_4 production and rumen fermentation characteristics associated with the reintroduction of protozoa into previously protozoa-free cattle and also assess whether CH_4 mitigation arising from NO_3 would be additive to that caused by the absence of protozoa. The protozoal population in previously defaunated heifers was established by day 7 and reached 7.01×10^5 cells/mL by day 21 comparable with that found by Morgavi *et al.* (2008) in sheep. These authors demonstrated that total protozoal population reached their peak at 12×10^5 cells/mL at 25 to 30 days after inoculation and then stabilised at 7.6×10^5 cells/mL from day 60. During the refaunation period there was a substantial increase in CH_4 production rate; this result was in accordance with the positive correlation between protozoa and CH_4 production found by Morgavi *et al.* (2010) and the fact methanogens that are normally attached to protozoa (Newbold *et al.* 1995) are responsible for 37% of

rumen CH_4 emissions (Finlay *et al.* 1994). The present study also showed that rumen fluid from defaunated heifers tended to have a lower CH_4 production *in vitro* than samples from refaunated heifers 7, 14 and 21 days after refaunation. This effect may not be exclusively a direct consequence of protozoa but also an indirect consequence of differences in bacterial and fungal populations in the presence of protozoa (Eugène *et al.* 2004a) and in some cases, an increase in activity of H_2 producers (Morgavi *et al.* 2012). Such compensatory changes in microbial populations leading to an unchanged VFA pattern may explain why the absence of protozoa has caused no significant changes in CH_4 emissions in defaunated animals as observed from some previous studies (Bird *et al.* 2008; Hegarty *et al.* 2008; Morgavi *et al.* 2012).

Effects of protozoa on rumen NH_3 concentrations are generally more consistent than effects on VFA concentration with the concentration of NH_3 lower in defaunated ruminants compared to faunated or refaunated ones in this and previous studies (Jouany *et al.* 1988; Eugène *et al.* 2004a; Santra *et al.* 2007a; Morgavi *et al.* 2012). Defaunation has sometimes increased total VFA concentration in defaunated sheep (Santra *et al.* 2007a) and weaner lambs (Santra and Karim 2002), but Hegarty *et al.* (2008) found total VFA was lower and the proportion of propionate was reduced in the protozoa-free lambs born from defaunated ewes. These authors suggested that effects of defaunation on reducing CH_4 production may be dependent upon fermentation shifting to a more propionate rich pattern in defaunated animals. This is consistent with defaunation normally increasing the proportion of propionate and decreasing the proportion of butyrate while concomitantly reducing CH_4 output (Eugène *et al.* 2004a; Morgavi *et al.* 2012). No differences between defaunated and refaunated heifers in concentration and

proportions of VFA were observed in these studies but the absence of protozoa still reduced CH_4 production, indicating that protozoal effects on methanogenesis are not just a consequence of increased partitioning of H_2 into propionate synthesis.

Importantly, the successive *in vitro* studies show that despite defaunation being completed 15 days before day 0; the rumen of defaunated heifers was not metabolically stable, with pH, total VFA, NH_3 and acetate percentage changing out to day 21 in Experiment 1. Little is known about rumen ecological stabilisation after defaunation, it was presumable in these studies that rumen ecology was stable within 50 days after defaunation and therefore was stable when the combined effects of NO_3 and defaunation were assessed in the Experiment 2.

Dietary NO_3 has been shown to offer a reliable and predictable strategy to mitigate CH_4 production from ruminants in both *in vitro* and *in vivo* studies. A review by Leng and Preston (2010) concluded that the use of NO_3 as a H_2 sink could reduce CH_4 production from 16-50%, depending on diets and the inclusion rate of NO_3. This is because approximately 2 mol of H_2 will be needed to convert NO_3 to nitrite and 6 mol of H_2 will be removed in order to reduce nitrite to NH_3 (Allison and Reddy 1984). The result from the Experiment 2 shows that CH_4 production was significantly lowered by addition of NO_3 in refaunated heifers 35 days after refaunation, confirming the potential for role of dietary NO_3 as a strategy to mitigate CH_4 emissions (Guo *et al.* 2009; Nolan *et al.* 2010; van Zijderveld *et al.* 2010; van Zijderveld *et al.* 2011). In addition, NO_3 reduced total gas production, total *in vitro* VFA concentrations and the proportion of butyrate in line with findings of Lin *et al.* (2011). The present study also indicated that

the combined effects of protozoal treatment and dietary NO_3 led to more than additive reduction in CH_4 production (19.11 mL) compared with the sum of the protozoal effect (3.52 mL) and the dietary NO_3 effect (9.38 mL).

5.5 Conclusion

Methane production was positively correlated with protozoal numbers in rumen fluid in the period following refaunation of defaunated heifers with protozoa (Experiment 1). The absence of protozoa reduced CH_4 production by 16% compared with refaunated heifers, and the combined effects of NO_3 and defaunation was synergistic in CH_4 mitigation (Experiment 2). Future research is needed to confirm these suggestions and gain better understandings the changes in gut fermentation, adaptation of methanogen and increased activity of some rumen microbes after defaunation and refaunation. *In-vivo* experiments need to be undertaken to gain a better understanding of the combined effects of defaunation and dietary NO_3 on CH_4 production in cattle.

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Chapter 6

Effects of defaunation and dietary coconut oil distillate on fermentation, digesta kinetics and methane production of Brahman heifers

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Abstract

A 2×2 factorial experiment was conducted to assess the effects of presence or absence of rumen protozoa and of dietary coconut oil distillate (COD) supplementation on rumen fermentation characteristics, digesta kinetics and methane (CH_4) production in Brahman heifers. Twelve Brahman heifers were selected to defaunate, with 6 being subsequently refaunated. After defaunation and refaunation, heifers were randomly allocated to COD supplement or no supplement treatments while fed an oaten chaff basal diet. Daily methane production (DMP; 94.17 v 104.72 g/d) and methane yield (MY; 19.45 v 21.64 g/dry matter intake) were reduced in defaunated heifers compared to refaunated heifers when measured 5 weeks after refaunation treatment ($P < 0.01$). Supplement of COD similarly reduced DMP and MY (89.36 v 109.53 g/d and 18.46 v 22.63 g/kg dry matter intake respectively; $P < 0.01$) and there were no significant interactions of defaunation and COD effects on rumen fermentation or CH_4 emissions. Concentration of volatile fatty acids and molar proportions of acetate, propionate and butyrate were not affected by defaunation or by COD. Microbial crude protein outflow was increased by defaunation ($P < 0.01$) in the absence of COD but was unaffected by defaunation in COD supplemented heifers. There was a tendency towards a greater average daily gain in defaunated heifers ($P = 0.09$), but COD did not increase average daily gain ($P > 0.05$). The results confirmed that defaunation and COD independently reduced enteric DMP even though the reduced emissions were achieved without altering rumen fermentation volatile fatty acid levels or gut digesta kinetics.

Keywords: Protozoa, methanogensis, fatty acids, cattle.

6.1 Introduction

Of the 87.4 Mt CO_2-e total greenhouse gas emissions released annually from Australia's agricultural sector, 64% are from enteric fermentation of livestock (Department of the Environment 2014). Enteric methane (CH_4) is not only Australia's largest agricultural emission source but also represents a loss of 5 to 7% of gross energy intake, equivalent to a CH_4 yield of 16 to 26 g CH_4/kg of dry matter consumed (Hristov *et al.* 2013). Reviews of the effects of enteric protozoa on digestion and productivity by ruminants have concluded that removal of rumen ciliate protozoa (defaunation) reduces enteric CH_4 emissions by 11% (Newbold *et al.* 2015) and increases average daily gain by 11% (Eugène *et al.* 2004a). Despite these average effects, there are many studies where animals with a stable protozoa-free rumen do not exhibit reduced CH_4 emissions. Defaunation did not change enteric CH_4 production 10 to 25 weeks post-treatment (Bird *et al.* 2008) and did not affect CH_4 production by lambs raised without protozoa from birth or from weaning (Hegarty *et al.* 2008). Although, defaunation reduces the number of methanogens in rumen fluid, it does not always reduce CH_4 production (Kumar *et al.* 2013). Therefore, evidence that CH_4 emissions are reduced in ruminants that have a stable long-term defaunated rumen remains unclear.

The use of medium-chain fatty acids to reduce methanogen and ciliate protozoal populations in the rumen has been shown as a potential strategy to reduce CH_4 production. Coconut oil (CO) is a rich source of medium-chain unsaturated fatty acids and feeding 3.5% and 7% CO reduced CH_4 production by 28% and 73% in sheep (Machmüller and Kreuzer 1999). Feeding 50 g CO/day significantly reduced CH_4 emissions without affecting the total tract dry matter digestion or energy retention

within sheep (Machmüller *et al.* 2003). Jordan *et al.* (2006) showed that feeding 250 g refined CO/day to beef heifers reduced CH_4 output by 18%, while dry matter intake was maintained and liveweight gain increased. However, no interaction between CO feeding and defaunation was observed for CH_4 production (Machmüller *et al.* 2003).

While studies of rumen ciliate protozoa effects on CH_4 production and performance of sheep have been recently published (Eugène *et al.* 2010; Morgavi *et al.* 2012; Zeitz *et al.* 2012), there is little data on defaunation of cattle. Therefore, this study sought to investigate the fermentation characteristics, digesta kinetics and CH_4 emissions in defaunated beef heifers compared to heifers with protozoa on a forage diet, and assess whether coconut oil distillate (COD) supplementation could further reduce CH_4 emissions from defaunated heifers.

6.2 Materials and methods

6.2.1 Animals and feeding

All protocols for treatment and care of the cattle were approved by the University of New England Animal Ethics Committee (AEC 13-054). Twelve purebred Brahman heifers (8 months of age) with an average liveweight (± s.e) of 280 ± 27 kg were obtained and defaunated. After this defaunation program, heifers were allocated to four experimental groups by stratified randomisation procedures based on liveweight to form a 2×2 factorial design (protozoa status either defaunated or refaunated; COD supplementation at 0 or 4.5%). A diet of 4.5 % COD and 4.5% molasses was prepared by sprinkling of the liquid COD and molasses onto a mix of 70% oaten and 21% lucerne chaff while the chaff was tossed in a rotary feed mixer (+COD; Table 6.1). As

COD was not highly palatable, molasses was added in the diet to enable consumption of 4.5% COD. The unsupplemented control diet (-COD; Table 6.1) was prepared as a straight mix of 70% oaten and 30% lucerne hay. All heifers had *ad libitum* access to the diets until 3 days prior to CH_4 measurement from day 15 to day 18 (Table 6.2), at which time feed intake was restricted to 80% of averaged *ad libitum* intake. Restricted feed intake continued during a measurement period of faeces collection for analysis of digesta kinetics from day 18 to day 22. Heifers were fed twice daily in two equal portions at 0930 and 1500 hours. Water was always available *ad libitum*. All heifers were weekly weighed to monitor liveweight and determined average daily gain (ADG).

6.2.2 Feed sampling and chemical analyses

Feed samples (~100 g) were collected before and after each mix of feed and stored at -20^{0}C. All samples were pooled and sub-samples were taken to analyse chemical composition (Table 6.1). Feed samples were analysed by the NSW DPI Feed Quality Service, Wagga Wagga Agriculture Institute, NSW, Australia. Crude protein was assessed by wet chemistry (AOAC 990.03 method), crude fat by petroleum ether extract, metabolizable energy by the AFIA 2.2R method, dry matter digestibility, digestible organic matter in the dry matter by wet chemistry (AFIA method 1.7R), acid detergent fibre and neutral detergent fibre by near-infrared spectroscopy (AFIA 2014).

Table 6.1 Composition of the diets and fatty acid profile of coconut oil distillate (g/100g dry matter).

Component	(+COD)*	(-COD)†
Dry matter (g/100g as fed)	88.1	88.8
Dry matter digestibility	60	60
Digestible organic matter in dry matter	60	58
Inorganic ash	8.0	8.0
Organic matter	92	92
Neutral detergent fibre	48	52
Acid detergent fibre	28	31
Crude protein	8.2	10.3
Metabolisable energy (MJ/kg)	10.0	8.9
Crude fat	5.0	1.3
Fatty acid profile (%) of COD		
C8:0	6.15	
C10:0	5.00	
C12:0	42.08	
C14:0	15.69	
C16:0	13.41	
C18:0	3.50	

**Oaten chaff (70%), lucerne chaff (21%), coconut oil distillate (4.5%), molasses (4.5%) fresh weight basis; †Oaten chaff (70%), lucerne chaff (30%) fresh weight basis.*

6.2.3 Defaunation of cattle

All heifers were acclimated to a diet consisting of oaten (70%) and lucerne (30%) chaff with initial COD from 0 to 4.5% for 18 days to supress protozoa. Initial COD treatment reduced the protozoal population from 3.91×10^5 cells/mL to 0.58×10^5 cells/mL of rumen fluid. The defaunation treatment was adapted from a protocol by Bird and Light (2013), with heifers fasted for 24 h and then orally dosed with sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol ESB/70AV, Albright and Wilson Australia Ltd, Melbourne) administered at 45 g/d in a 10% v/v solution to remove protozoa.

Heifers were dosed on three consecutive days and feed was withheld during this period. Animals required 15 days to recover the voluntary intake observed prior to treatment. The three day dosing with Empicol was repeated commencing 15 days after the first dosing. Weekly rumen fluid samples were collected from all heifers for protozoa enumeration commencing a further 15 days after the second drenching program.

Table 6.2 Experimental schedule for the defaunation, refaunation and data measurements.

	Day	Activity
Defaunation	-69	Coconut oil distillate (COD) feeding period: 18 day following 8 day adaptation
	-51	First three day defaunation protocol and recovery period commencing on day 51
	-36	Second three day defaunation protocol and recovery period commencing on day 36
Refaunation	-21	Six heifers were inoculated with rumen fluid from cannulated cattle to refaunate
	-21, -14, -7, 0	Protozoa check in both groups of defaunation and refaunation, rumen VFA and ammonia sampling
Comparative study	0	Start of 2x2 study (COD supplementation at 0 and 4.5%; protozoa status either defaunated or refaunated)
	15 - 18	Methane production measured, rumen protozoa, VFA and NH_3 sampling
	18 - 22	Digesta kinetics measured, spot urine samples, rumen protozoa, VFA and NH_3 sampling

6.2.4 Refaunation of cattle

All heifers had recovered their intake and wellbeing 15 days after the second defaunation program, and their rumen fluid was visually observed to be free of protozoa. Six heifers were selected for refaunation by stratified randomisation based on liveweight. A single oral dose (total of 500 mL/heifer) of a mixed rumen fluid collected from two cannulated faunated cattle grazing pasture was used to refaunate 6 heifers. The protozoal population in the inoculum (3.42×10^5 cells/mL) consisted of large holotrichs (0.13×10^5 cells/mL), small holotrichs (0.5×10^5 cells/mL) and small entodiniomorphs (2.79×10^5 cells/mL).

6.2.5 Rumen fluid sampling, ammonia, volatile fatty acid concentrations, and protozoal enumeration

Samples of rumen fluid were collected weekly by oesophageal intubation commencing 15 days after the second defaunation program from defaunated animals to confirm the sustained protozoa free status during the experimental period. Additional samples from refaunated heifers were also collected to monitor the protozoal growth immediately after inoculation. When collecting rumen fluid, rumen pH was measured immediately upon sampling using a portable pH meter (Orion 230 Aplus, Thermo scientific, Beverly, MA, USA). A 15 mL subsample was placed in wide-neck McCartney bottle acidified with 0.25 mL of 18 M sulphuric acid and stored at -20^0C for volatile fatty acid (VFA) and ammonia (NH_3) analyses. A 4 mL subsample was placed in wide-neck McCartney bottle containing 16 mL of formaldehyde-saline (4% formalin v/v) for protozoa enumeration and stored at room temperature for the visual enumeration of ciliate

protozoa. Protozoa were stained prior to counting using an adaption of the procedure of Dehority (1984). Protozoa were counted using a Fuchs–Rosenthal optic counting chamber (0.0625 mm^2 and 0.2 mm of depth). The protozoa were differentiated into large (>100 µm) and small (<100 µm) holotrich and entodiniomorph grouping. Concentration of VFAs was determined by gas chromatography (Nolan *et al.* 2010) using a SMARTGAS Varian CP 3800 Gas Chromatograph (Varian Inc. Palo Alto, California USA) and NH_3 concentration was analysed using a modified Berthelot reaction using a continuous flow analyser (San^{++}, Skalar, Breda, The Netherlands).

6.2.6 Methane production measurement

Daily methane production (DMP, g CH_4/day) was measured in respiration chambers over 2×22 h consecutive period (Hegarty *et al.* 2012). Heifers were placed in their chambers by 1100 hours, with their feed and water available inside the chambers. The chambers were opened to collect refusals and supply fresh feed at 0900 hours the following day and only resealed at 1100 hours to commence the second 22 h of measurement until 0900 hours the following day.

Methane recovery through each chamber was quantified immediately before and after the experimental period. Pure CH_4 was infused into each chamber at a known rate using a mass flow meter (Smart Trak 2 Series 100, Sierra Instruments, Monterey). The concentration of CH_4 reached a plateau in the chamber after 60 mins. Knowing the inflow rate of CH_4 (CH_4 infusion rate) and the chamber outflow rate (air flow rate through the chamber) an expected plateau value for each chamber could be calculated. A value for CH_4 recovery was obtained (89-100%) from the ratio of the measured

plateau CH_4 concentration and expected methane plateau value and DMP corrected accordingly. Methane yield (MY) was calculated as DMP divided by dry matter intake (DMI).

6.2.7 Digesta kinetics and estimation of microbial protein supply

A 5-day collection of faecal output was conducted to determine digesta kinetic and dry matter digestibility (DMD). The mean retention time (MRT, h) of digesta was estimated in all heifers over 5 days by reference to faecal excretion of a dosed particle-phase marker (50 g per heifer of Cr-mordanted NDF from oaten chaff) prepared in accordance with Udén *et al.* (1980), and a liquid-phase marker (60 g per heifer of Co-EDTA from AVA Chemicals Pty Ltd. Mumbai, India in 250 mL of Milli-Q water). Dissolved Co-EDTA was administered via intubation directly into the rumen as a single dose while the Cr-mordanted fibre was offered to each heifer with 100 g of lucerne chaff immediately prior to the morning feed. Faecal samples were collected at 6 h after administration of the liquid marker, following by every 3 h for the next 48 h, every 8 h for the next 24 h and every 12 h for the next 24 h.

Samples were analysed Cr and Co concentrations (Barnett *et al.* 2016) using portable X-ray fluorescence spectroscopy (Bruker Tracer III-V pXRF, Bruker Corp, MA USA). Analysis of digesta kinetics was undertaken using non-linear curve fitting algorithms of WinSAAM (Aharoni *et al.* 1999).

During faecal marker collection, spot samples of urine were collected from all heifers. Urine samples in 50 mL plastic bottles containing 5% H_2SO_4 were stored at -20 ^{0}C until analysis. Urine allantoin and creatinine concentrations were determined (IAEA, 1997).

Microbial crude protein (MCP) outflow was estimated in spot urine samples as calculated by the equations of Chen *et al.* (2004).

6.2.8 Statistical analyses

Data was statistically analysed using SAS 9.0 (SAS Institute, Cary, NC). Data from rumen fermentation characteristics, digesta kinetics, MCP outflow, DMP and MY were subject to analysis of variance in PROC GLM with factors being protozoa, COD and protozoa × COD interaction. For analysis of ADG, the model used the initial liveweight as a covariate. Homogeneity of variance and normal distribution were tested using PROC UNIVARIATE before statistical analysis. Means were analysed using the least squares means (LSMEANS) procedure. A probability of < 5% was considered to be statistically significant.

6.3 Results

6.3.1 Protozoal populations

Defaunated heifers remained protozoa-free throughout the study. In refaunated heifers, the total numbers of protozoa after refaunation reached 3.70×10^5/mL by day 7 and doubled by day 21 (7.38×10^5/mL), which was day 0 of COD feeding period (Table 6.3). Small entodiniomorphs were predominant in the population of rumen protozoa, ranging from 82 to 94% of the total. The total number of protozoa and numbers of small entodiniomorphs in refaunated heifers after 22 days of feeding COD were significantly reduced by COD (Table 6.3) such that total numbers of protozoa and of small entodiniomorphs were reduced by COD by 84% and 82% respectively ($P < 0.05$). Large

holotrichs were not affected by COD while small holotrichs were not detected after day 22 feeding COD. Large entodiniomorphs were not observed in the inoculum or in any rumen fluid samples of refaunated heifers.

6.3.2 Rumen pH, volatile fatty acid and ammonia concentrations

Defaunation reduced rumen pH ($P = 0.03$) while COD supplement did not affect rumen pH ($P > 0.05$; Table 6.4). Concentrations of VFA and molar proportions of acetate, propionate, butyrate and the molar ratio of acetate to propionate were not significantly affected by defaunation or by COD. There were no interactions between protozoal treatments and dietary COD on concentrations of VFA or their molar proportions ($P > 0.05$). Defaunation and COD significantly reduced ruminal NH_3-N concentration by 39% and 61% respectively ($P < 0.05$) and an interaction between defaunation and COD was found ($P < 0.01$) such that refaunation raised ruminal NH_3-N concentration in the absence of COD, but did not affect NH_3 when COD was included (Figure 6.1).

Table 6.3 Enumeration of protozoa following refaunation in heifers and fed a diet containing either 4.5% coconut oil distillate (+COD) or nil (-COD).

Parameters	Refaunated heifers				s.e.m	*P*-Values		
	Initial (d 0)		Final (d 22)			COD	Day	COD × Day
	-COD	+COD	-COD	+COD				
Total protozoa ($\times 10^5$/mL)	7.04	7.72	6.88	1.25	1.00	0.01	0.04	0.02
Large holotrich	0.06	0.19	0.17	0.10	0.11	1.00	0.81	0.34
Small holotrich	0.47	1.17	0.61	0	0.10	<.01	0.68	<.01
Small entodiniomorph	6.52	6.36	6.10	1.17	0.92	0.02	0.02	0.03

Standard error of the mean (s.e.m).

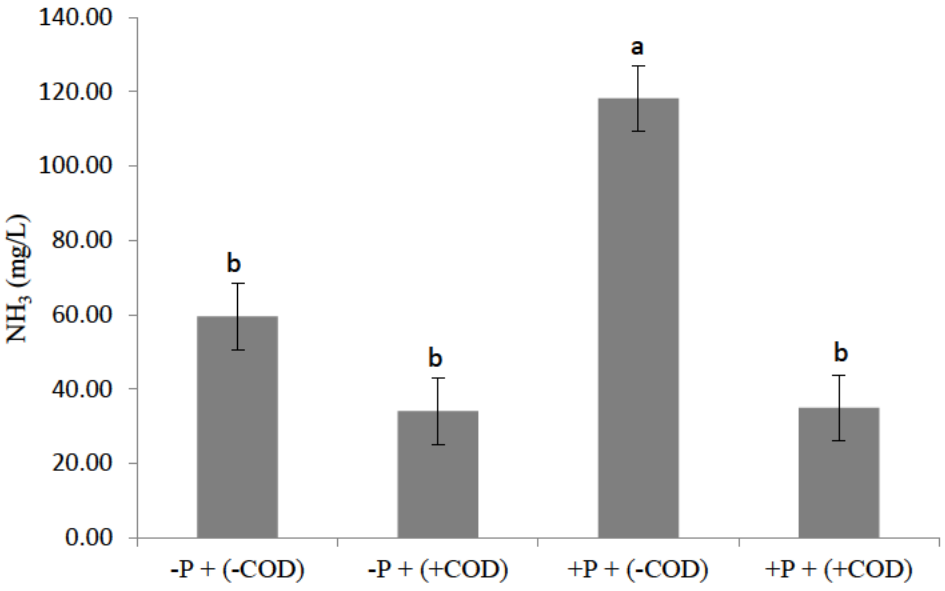

Figure 6.1 Relationship between protozoa (-P defaunated; +P refaunated) and COD supplement (-COD, unsupplemented; +COD, supplemented) on ruminal NH_3-N concentration. Error bars indicate s.e and a common suffix above error bars indicate non-significant difference.

Table 6.4 Physiological and rumen fermentation characteristics as influenced by the presence (+P) or absence of protozoa (-P) or coconut oil distillate (±COD) supplementation.

Parameter	Treatment				SEM	*P*-Values		
	Protozoa		COD			P	COD	P× COD
	-P	+P	-COD	+COD				
Rumen pH	6.29	6.50	6.40	6.38	0.06	0.03	0.82	0.24
NH_3-N (mg/L)	46.83	76.60	88.90	34.53	6.36	0.01	<.01	0.01
Total VFA (mM/L)	49.30	42.53	44.16	48.03	4.05	0.18	0.56	0.55
Acetate (molar %)	76.19	75.54	76.05	75.67	1.06	0.67	0.81	0.29
Propionate (molar %)	14.98	13.64	13.83	14.79	0.62	0.14	0.29	0.13
Butyrate (molar %)	6.75	7.58	6.67	7.66	0.53	0.28	0.20	0.87
Acetate / propionate ratio	5.23	5.72	5.69	5.25	0.32	0.29	0.33	0.11
DMI (kg/day)*	5.36	5.45	6.25	4.63	0.20	0.24	<.01	0.69
DMD (%)†	46.38	45.05	49.99	41.44	3.28	0.78	0.10	0.06
DMP (g CH_4/day)	94.17	104.72	109.53	89.36	3.45	<.01	<.01	0.33
MY (g CH_4/kg DMI)‡	19.45	21.64	22.63	18.46	0.71	<.01	<.01	0.32
MI (g CH_4/kg ADG)	125.3	245.2	223.0	147.5	60.54	0.08	0.24	0.20
ADG (kg/day)§	0.84	0.54	0.71	0.67	0.12	0.09	0.69	0.25
Final LW (kg)	313.7	301.0	308.2	306.5	7.70	0.13	0.80	0.27
FCR (g FI/g ADG)¶	7.84	14.65	13.95	8.54	2.39	0.07	0.14	0.16
MCP outflow (g/day)	85.36	66.21	80.65	70.92	6.26	<.01	0.13	0.02
Rumen soluble MRT (h)	16.52	15.40	14.72	17.20	2.27	0.64	0.31	0.46
Hindgut soluble MRT (h)	8.64	7.60	8.24	8.00	0.75	0.20	0.76	0.36

*Standard error of the mean (s.e.m); *Dry matter intake (DMI) was calculated when cattle were on ad libitum fed; †Dry matter digestibility (DMD) was estimated based on soluble marker (Co-EDTA); ‡kg DMI was calculated when heifers were on restricted intake;* §Average daily gain (*ADG) was calculated as (LW at d-21- final LW at d 22)/43 days; Daily methane production (DMP); Methane yield (MY); Methane intensity (MI).*¶*Feed conversion ratio (g feed intake/g ADG).*

6.3.3 Methane emissions

Daily methane production (g CH_4/d) and MY (g CH_4/kg DMI) were significantly reduced by defaunation (10%) and COD (18%), respectively ($P < 0.01$; Table 6.4). Combination of defaunation with COD supplement did not cause interactions in DMP

or MY, indicating that the effects were at least additive. When CH_4 intensity (MI; g CH_4/kg ADG) was calculated using ADG over 43 days from defaunation to the end of the study, defaunation tended to reduce MI ($P = 0.08$), but supplementation of COD did not change MI ($P = 0.24$).

6.3.4 Dry matter intake, digestibility, digesta kinetics, microbial protein outflow and liveweight change

The presence or absence of rumen protozoa did not affect DMI, but COD addition reduced DMI ($P < 0.05$) as the DMI was 26% lower in heifers with COD compared to heifers without COD. Protozoal treatment and dietary COD did not affect DMD or the rumen or hindgut MRT of liquid digesta ($P > 0.05$; Table 6.4). Rumen and hindgut particle MRT were unable to be estimated due to heifers being slow to consume the Cr-mordanted NDF dose, making fitting of dilution curve difficult.

Defaunation increased MCP outflow by 22% ($P < 0.01$; Table 6.4), but MCP outflow was not affected by COD supplementation. There was an interaction between defaunation and COD on MCP outflow ($P = 0.02$, Figure 6.2), showing reduced MCP outflow in defaunated heifers when COD was supplemented, but MCP outflow was unaffected by COD in refaunated heifers which all had a low MCP outflow. There was a positive correlation between MCP outflow and ADG such that higher MCP outflow was associated with higher ADG (ADG = -0.34 + 0.014 MCP outflow, $r^2 = 0.67$, $P = 0.02$). Defaunated heifers tended to have a greater ADG ($P = 0.09$) and a lower feed conversion ratio (FCR, $P = 0.07$) compared to refaunated heifers, while COD

supplementation did not cause changes in final liveweight, ADG or FCR in heifers (Table 6.4).

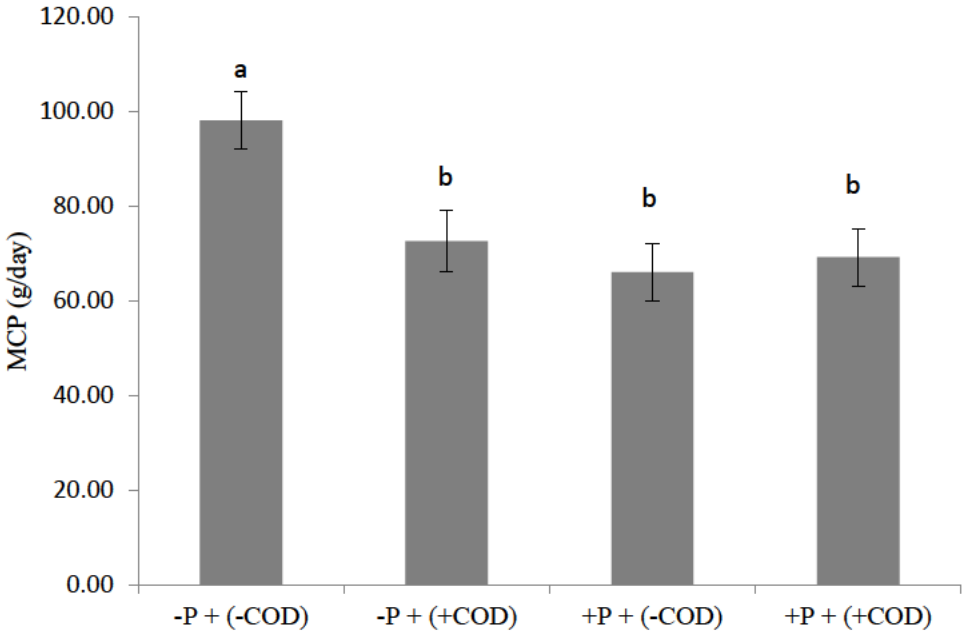

Figure 6.2 Relationship between absence/presence of protozoa (-/+P) and of coconut oil distillate supplement (-/+COD) on microbial crude protein (MCP) outflow. Error bars indicate s.e and a common suffix above error bars indicate non-significant difference.

6.4 Discussion

This study investigated the effect of the absence of protozoa and inclusion of a medium-chain fatty acid (MCFA) supplement on fermentation and CH_4 production of cattle. It has shown that both defaunation and addition of COD as a source of MCFA can reduce enteric CH_4 emissions.

In sheep, defaunation has been successfully achieved with sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol ESB/70AV, Albright and Wilson Australia Ltd, Melbourne) (Bird *et al.* 2008; Hegarty *et al.* 2008), but there are very few reports of successful defaunation of cattle (Bird and Light 2013), probably because omasal ciliate protozoa are difficult to eliminate in cattle (Towne and Nagaraja 1990). A defaunation protocol modified from that used in sheep was developed, with preliminary addition of COD rich in lauric acid (42%) to the diet for 18 days prior to treatment with Empicol. This COD treatment reduced the rumen protozoal population by 85%, and following two treatments of Empicol, rendered cattle without detectable levels of rumen protozoa.

6.4.1 Protozoal population in refaunated heifers after inoculation and effect of coconut oil distillate on protozoal population

The protozoal population in heifers after refaunation from a previously defaunated state was well established by day 7 and reached 7.38×10^5 cells/mL by day 21, comparable with that found by Sénaud *et al.* (1995) who re-inoculated defaunated rumen with *Isotricha* and ciliates of mixed fauna and found that the maximum concentration of protozoa was reached 9 to 17 days after inoculation. The maximum population then decreased for 2-3 days before stabilising. Morgavi *et al.* (2008) also demonstrated that total protozoal populations reached their peak at 12×10^5 cells/mL by 25 to 30 days after inoculation and then stabilised at 7.6×10^5 cells/mL from day 60.

Capric acid (C10:0), lauric acid (C12:0) and myristic acid (C14:0) show strong protozoal toxicity and are useful rumen defaunating agents (Matsumoto *et al.* 1991).

The present study showed that total numbers of rumen protozoa were reduced by 81% after 22 days of feeding COD (containing 42% lauric acid) when included at 4.5% of the diet. Matsumoto *et al.* (1991) observed the rumen protozoa, except *Entodinium spp*. were undetectable after 3 days feeding of 30 g of hydrated CO containing 52% lauric acid. Feeding 250g of refined CO to beef heifers reduced total protozoa by 62% (Jordan *et al.* 2006) and protozoal populations in beef heifers were decreased by 63% and 80% by 300 g/d CO after 45 and 75 days, respectively (Lovett *et al.* 2003). Machmüller, (2006) observed a reduction in rumen protozoa by 88 and 97% when feeding sheep with 3.5 and 7% CO respectively. This suppressive effect of CO on rumen protozoa even persisted 5 weeks after finishing feeding sheep with CO (Sutton *et al.* 1983).

6.4.2 Effects of defaunation treatment

The effects of defaunation on VFA concentration and the molar proportions of VFA are not entirely consistent in the literature (Jouany *et al.* 1988; Williams and Coleman 1992; Eugène *et al.* 2004a; Newbold *et al.* 2015), and in this study defaunation did not cause changes in total VFA or molar proportions of acetate, propionate and butyrate. Defaunation sometimes increased total VFA concentration in defaunated sheep (Santra *et al.* 2007a) and weaner lambs (Santra and Karim 2002), but Hegarty *et al.* (2008) reported that animals with protozoa had higher concentrations of total VFA compared with defaunated animals. Molar proportions of VFA were also inconsistently affected by defaunation as butyrate and acetate proportion were increased (Machmüller *et al.* 2003; Bird *et al.* 2008) and proportion of propionate was decreased (Machmüller *et al.* 2003; Hegarty *et al.* 2008). A higher proportion of acetate and lower proportion of

propionate in the VFA of defaunated animals was a common finding by Bird, (1982) when animals were fed low-quality diets. These inconsistent effects of defaunation on VFA concentration and molar proportions may reflect variable effects of defaunation on the bacterial population in defaunated rumens. Reviews of literature by Jouany *et al.* (1988) concluded that defaunation increased numbers of bacteria, which induced changes in digestion and fermentation due to bacterial species distribution and bacterial composition had been changed after defaunation (Ozutsumi *et al.* 2005).

In the present study, the reduced DMP of defaunated heifers was not associated with increased molar proportions of propionate, which contrasted to a study by Eugène *et al.* (2004a). Morgavi *et al.* (2012), however, reported a negative correlation between H_2 concentration and CH_4 production, such that less production of CH_4 resulted in a high concentration of dissolved H_2, but propionate production did not increase. Accumulation of H_2 in association with lower CH_4 production may reflect reduced capacity to utilise H_2 by microbes in the defaunated rumen and it is possible that reductive acetogenic bacteria in the rumen (Joblin 1999) could potentially convert accumulated H_2 and CO_2 into acetate when H_2 partial pressure is raised (Fonty et al. 2007; Ungerfeld 2013). As H_2 concentration and methanogen populations were not measured in this study, it is not possible to confirm that inhibition of methanogenesis caused accumulation of H_2 or induced reductive acetogenesis.

Methanogens, which exist as endo- and ecto-symbionts with ciliate protozoa (Finlay *et al.* 1994; Tokura *et al.* 1997), had been estimated to accout for 37% of CH_4 production (Finlay *et al.* 1994) and the proportions of methanogens in the total bacterial population

were lower in association with a 26% lower CH_4 emissions from protozoa-free lambs compared to faunated lambs (McAllister and Newbold 2008). In addition, the archaeal community of methanogens in liquid and solid rumen contents were similar in faunated wethers, but a lower proportion of methanogens in the liquid phase was associated with defaunation (Morgavi *et al.* 2012). However, Mosoni *et al.* (2011) observed a 20% reduction in CH_4 emissions in long-term (2 year) and short-term (10 week) defaunation, but methanogens per gram of dry matter of rumen content increased while the diversity of dominant methanogenic community was not changed. Therefore, it may not be reasonable to attribute the reduced CH_4 production from defaunation to a loss of methanogens (Morgavi *et al.* 2012). It is hypothesised that loss of ciliate-associated methanogens in the defaunated rumen reduced CH_4 production but may have induced an increase in other populations of micro-organism, with a lower prevalence of H_2 producers.

Removing protozoa from the rumen may allow a proliferation of rumen bacteria, leading to increased uptake of NH_3 by bacteria for protein synthesis and less protein being degraded by protozoa (Williams and Coleman 1992). Decreases in NH_3 concentration in defaunated animals compared to faunated or refaunated animals were observed in this study and confirm previous assessments (Jouany *et al.* 1988; Eugène *et al.* 2004a; Santra *et al.* 2007a; Morgavi *et al.* 2012; Newbold *et al.* 2015). Less ruminal catabolism of engulfed feed-protein and bacteria occurs in the absence of protozoa, leading to an increase in the supply of protein to the duodenum (Bird and Leng 1978; Jouany 1996). The present study showed 22% increased MCP outflow in defaunated heifers, which was consistent with previous studies. The increased MCP outflow was

associated with a 9-35% increased ADG in defaunated animals given high or low-quality forage diets (Bird 1989). There was a tendency for defaunated heifers to have a greater ADG, and this could only be ascribed to the increased MCP supply and/or reduced loss of digested energy in CH_4. Heifers in this experiment received oaten chaff based diet providing 10 g of CP per MJ of ME, which theoretically met the microbial protein yield required for growing cattle (CSIRO 2007). However, defaunated heifers had a decreased NH_3 concentration to below 50 mgN/L, which may have been insufficient to maximise microbial synthesis in the rumen (Satter and Slyter 1974). This could explain why defaunation did not more strongly increase ADG of animals given forage based diets in this study, although defaunation increased MCP outflow.

6.4.3 Effects of coconut oil distillate supplementation

The 20% and 24% reduction in DMP and MY when refaunated heifers received 4.5% COD (200 g/day) in this study was consistent with previous assessments (Lovett *et al.* 2003; Machmüller *et al.* 2003; Jordan *et al.* 2006; Machmüller 2006; Patra 2014). Jordan *et al.* (2006) reported a decrease in 18% CH_4 production by beef heifers that received 250 g/day of refined CO. The reduced CH_4 emissions by COD from this study were not associated with changes in total VFA or molar proportions of acetate or propionate. However, the relationship between feeding ruminants fat and the proportions of VFA associated with CH_4 production is not consistent with published data. Dietary fat decreased CH_4 production linearly by 4.3% per percentage of fat inclusion and tended to shift the proportions of VFA to greater propionate and less butyrate production in the study of Patra, (2014). In contrast, results from this study

agreed with Hristov *et al.* (2009) who reported no differences in total VFA when feeding lactating cows with lauric acid and CO. Methane production was reduced by 60% by CO, but the proportion of propionate was unchanged. In the same study by Hristov *et al.* (2009), lauric acid increased the proportion of propionate and decreased the proportion of butyrate, but CH_4 production was not reduced. In contrast, an *in vitro* study by Machmüller *et al.* (2002) showed that lauric acid reduced CH_4 production (mmol per gram organic matter degraded) by 76%, but did not affect total VFA or molar proportions of VFA. The reduced CH_4 production by MCFA in this and previous studies was most likely a result of reduced rumen protozoa and methanogens (Dohme *et al.* 2000; Liu *et al.* 2011) and also reduced butyrate producing bacterial populations such as *Butyrivibrio fibrisolvens* being inhibited by MCFA (Hristov *et al.* 2009).

Supplementation of COD reduced NH_3 concentrations which may have been a consequence of the rumen protozoa population being reduced by 81% in COD supplemented refaunated heifers that could indirectly affect deamination activity of rumen the rumen biota. This reduced population of protozoa may allow a compensatory increase in bacterial population, leading to more NH_3 utilisation by bacteria for protein synthesis (Machmüller and Kreuzer 1999) which, together with less degradation of dietary protein in the rumen (Jouany and Ushida 1999; Zeitz *et al.* 2012) would both reduce NH_3 accumulation. Sutton *et al.* (1983) found greater microbial synthesis in sheep supplemented with CO, however, the present study and a study of Machmüller and Kreuzer (1999) showed that microbial protein outflow was not increased by CO supplementation. This is because CO not only supresses the protozoal population but

also suppresses rumen bacteria (Dohme *et al.* 1999), which probably constrains microbial synthesis in the rumen.

Consistent with other studies (Sutton et al. 1983; Machmüller and Kreuzer 1999; Lovett et al. 2003; Hollmann et al. 2012), this study found 26% reduction in DMI in heifers given COD, thus the reduced CH_4 emissions were associated with an undesirable reduction in feed intake. This reduction in DMI was probably due to high level of lauric acid (42%) and myristic acid (15%) in COD as lauric and myristic acids depress feed intake (Dohme et al. 2001; Hristov et al. 2011). The effect of COD on DMD, digesta kinetics and ADG was inconsistent with literature (Sutton et al. 1983; Machmüller and Kreuzer 1999; Hollmann et al. 2012) who showed CO reduced ruminal fibre degradation and NDF digestion, which was probably due to the inhibited fibrolytic bacteria by dietary fat (Nagaraja et al. 1997). However, Machmüller et al. (2003) fed sheep 50g CO/kg dry matter and showed no adverse effects on the total tract dry matter digestion or energy retention. Jordan et al. (2006) also showed that feeding 250g/d of refined CO to beef heifers maintained intake and improved animal performance. Supplementation with COD in this study reduced DMI and NH3 concentration, but total VFA concentration and ADG were not affected, which probably reflects the COD increasing the average energy content of the diet consumed.

6.5 Conclusion

This experiment confirms that defaunation of the rumen and supplementation with COD independently reduces CH_4 emissions of cattle. The effects of defaunation in combination with COD in reducing DMP and MY were additive. However, the reduced emissions achieved by defaunation and supplementation with COD occurred without altering total VFA, the proportions of propionate and acetate, gut digesta kinetics or ADG. Further work is required to fully understand the effects of defaunation and COD supplementation on ruminal fermentation, gut digesta kinetics and CH_4 emissions.

Higher Degree Research Thesis by Publication

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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	Author's Name (please print clearly)	**% of contribution**
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Chapter 7

Distribution of ciliate protozoa populations in the rumen, reticulum, and omasum of Angus heifers

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Abstract

Ruminal, reticular and omasal contents and tissues were collected from Angus heifers (n = 8) at slaughter to determine the total protozoal population and its distribution within different parts of cattle foregut. The majority of protozoa at slaughter (99%) were present in free digesta not adhering to the gut wall (1.0%). The populations in the reticular and omasal digesta, while much smaller than (~5% of) that in the rumen, were of similar size to each other. The omasum surface, however, provides sequestration for a similar number of protozoa than does the entire rumen surface, indicating that the omasum may be an important reservoir for protozoa, especially entodiniomorphs. This is a likely reason why there are so few reports of cattle sustaining a protozoa-free rumen for a prolonged period.

Keywords: ciliate protozoa, foregut, cattle

7.1 Introduction

Removal of protozoa from the rumen (defaunation) increases bacterial biomass and increases flow of protein into the duodenum (Bird and Leng 1978; Jouany 1996), which is associated with a 9-35% increase in growth rate of defaunated relative to faunated ruminants (Bird 1989). Defaunation can also decrease enteric methane production (Kreuzer *et al.* 1986; Hegarty 1999; Morgavi *et al.* 2008) by eliminating methanogens that exist as endo- and ecto-symbionts with ciliate protozoa (Finlay *et al.* 1994) and by changing the molar proportions of VFA to a greater proportion of propionate and lesser proportion of butyrate (Eugène *et al.* 2004a). However, available techniques to completely remove protozoa are severe, possibly due to the difficulty in eliminating all

omasal protozoa, which are thought to migrate back into the rumen after ruminal defaunation is complete (Towne and Nagaraja 1990). Having shown there was merit in defaunation of cattle (Chapter 6) but great difficulty which was suggested as being caused by survival of an omasal population which resupplied the rumen, this study aimed to quantify resident ciliate protozoa populations both in the digesta and on the surface of the reticulum, rumen and omasum of Angus cattle.

7.2 Materials and methods

7.2.1 Animals, feed and sampling

All protocols for treatment and care of the cattle were approved by the University of New England Animal Ethics Committee (AEC 14-117). Angus heifers (n = 8; 226 ± 11.8 kg; 8 months of age) were offered 12 kg/head/day of a chaffed lucerne cereal hay mix (9.7 MJ ME/kg DM and 14.3% CP, Table 7.1) with the feed offered twice daily in two equal portions.

Samples of rumen fluid were collected every 2 weeks from each heifer before feeding using oesophageal intubation for protozoal enumeration from day 0 to day 42. On day 45, heifers were transported by truck 30 min to a local abattoir and killed the same morning with no feed being offered. Immediately after slaughter and evisceration, the reticulum and omasum were located and tied off to avoid flow of digesta within the foregut. The complete reticulum, rumen and omasum with and without digesta were weighed individually to determine weights of digesta contained, and tissue weight of each organ. Digesta from each organ was thoroughly mixed and approximately 20 g sub-samples were collected in 25 mL open ended syringes with the tops cut off. The

liquid was then squeezed out by placing a doubled layer of cheese-cloth over the open end then pushing the plunger into the barrel of the syringe. The pH of the resulting strained liquid (liquid fraction) was immediately measured using a portable pH meter (Orion 230 Aplus, Thermo scientific, Beverly, MA, USA) and then the liquid samples were preserved in pre-weighed containers containing formaldehyde-saline (4% formalin v/v; 0.9% NaCl w/v). The particulate digesta retained on the cheese-cloth (solid fraction) was also preserved in pre-weighed containers containing formaldehyde-saline. The containers were later re-weighed to determine weights of liquid and solid samples. Gut tissue samples were cut from each organ where the locations were identically located in each animal. Samples of gut tissues were fixed on plastic boards (3.5×4.5 cm) and then the samples were gently rinsed in clean water to wash off the trapped digesta before being preserved in pre-weighed containers with 10% (v/v) formalin to enable adherent protozoa to be counted. The containers were later re-weighed to determine the weight of gut tissue preserved.

Table 7.1 Chemical composition of the lucerne cereal hay mix (g/100g dry matter).

Component	Lucerne cereal hay mix
Dry matter (in feed as-fed)	88.7
Dry matter digestibility	69
Digestible organic matter	67
Organic matter	90
Neutral detergent fibre	42
Acid detergent fibre	31
Crude protein	14.3
Crude fat	1.4
Metabolisable energy (MJ/kg DM)	9.7

7.2.2 Sample processing and protozoal enumeration

A subsample (1.0 mL) of preserved liquid fraction was pipetted into a test tube. Two drops (0.05 mL) of brilliant green (2.0 g of brilliant green dye and 2.0 mL of glacial acetic acid diluted to 100 mL with distilled water) were added (Dehority 1984). The contents were mixed and allowed to stand overnight before counting of protozoal cells by microscopy.

A portion of each preserved sample of the digesta 'solid' fraction was further diluted with formaldehyde-saline, vortexed and then sonicated for 5 min to remove adherent protozoa. The homogenized preserved 'solids' samples were squeezed out through a doubled layer of cheese-cloth to separate liquid and solid fractions. A subsample (1.0

mL) of liquid was pipetted into a test tube to be stained with brilliant green. The solid content was placed in a 25 mL beaker to be thoroughly mixed and then 1.0 g of subsample was placed in a test tube. Three drops (0.075 mL) of brilliant green were added and vortexed to ensure thorough mixing. The contents were allowed to stand overnight. The preserved gut tissue samples were placed in a 50 mL beaker, thoroughly mixed and sonicated for 15 min to release adherent protozoa from the tissue. A 1.0 mL sample of sonicated preserved liquid fraction was pipetted into a test tube to be stained with brilliant green and allowed to stand overnight.

After staining, a portion of each stained sample was diluted with 30% glycerol, resulting in 1:20 dilution of the original sample. Protozoa were counted using a Fuchs–Rosenthal optic counting chamber (0.0625 mm^2 and 0.2 mm of depth). The protozoa were differentiated into large (>100 μm) and small (<100 μm) holotrich and entodiniomorph groupings. Total protozoal populations in, or on an organ's surface, were estimated as the product of weight of digesta (or weight of tissue) times the protozoa/g of digesta or protozoa/g of rinsed gut tissue for that organ.

7.2.3 Statistical analyses

Data were subject to analysis of variance (PROC GLM) using SAS 9.0 (SAS Institute, Cary, NC). Protozoa counts were log-transformed to meet homogeneity of variance and normal distribution criteria using PROC UNIVARIATE before statistical analysis. Least significant differences were used for means separation ($P < 0.05$).

7.3 Results

Rumen protozoal concentrations (cells/mL) monitored over 42 days prior to slaughter (Figure 7.1) showed protozoal concentrations had increased after initial introduction to the mixed lucerne and cereal chaff diet (a 48% increase over first 14 days; 1.39×10^5 v 2.68×10^5; $P < 0.05$). There were no significant differences in protozoal concentrations from day 14 to day 42 ($P > 0.05$), indicating that concentrations of rumen protozoa were stable in the weeks leading up to slaughter. Protozoal concentrations from the strained rumen fluid at slaughter on day 45 were similar to that of samples collected on day 0, 14, 28 and 42 by oesophageal intubation (Figure 7.1; $P > 0.05$). Small entodiniomorphs were dominant in the rumen fluid, ranging from 47 to 65% of the total protozoa (Figure 7.1).

The pH of digesta did not differ between reticulum, rumen and omasum. The rumen had the largest mass of digesta of all the forestomachs (Table 7.2). The total number of protozoa in the rumen was higher than in the reticulum and omasum. Although the quantity of digesta in the reticulum was smaller than in the omasum, the total number of protozoa was similar in both reticulum and omasum.

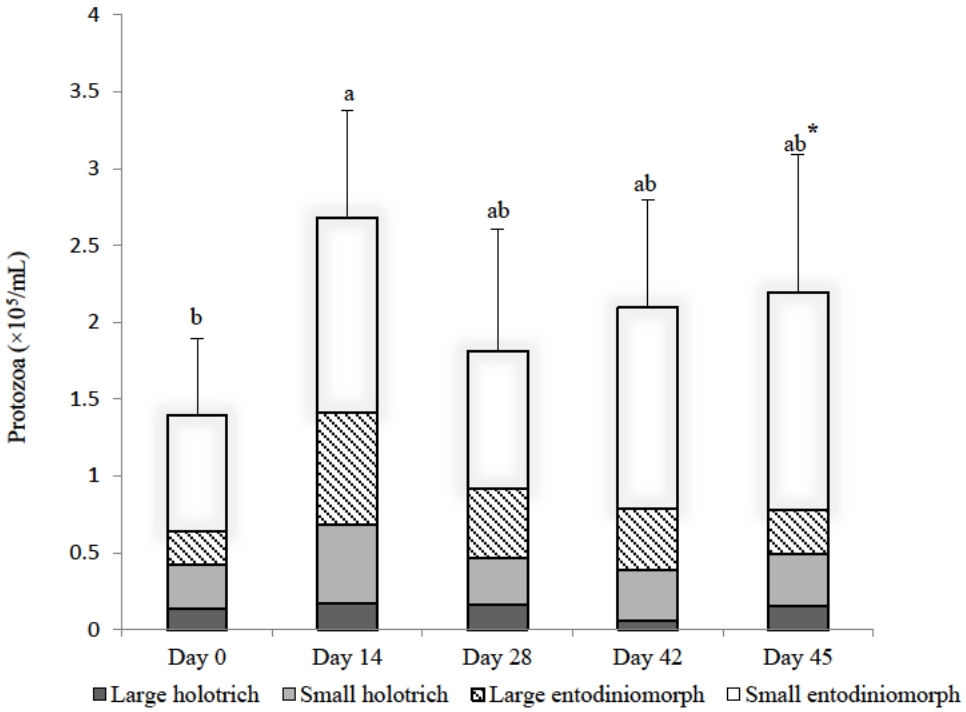

Figure 7.1 Protozoal population densities and proportions of main protozoal types (cells/mL) in rumen fluid from Angus heifers receiving lucerne cereal hay mix over a period of 42 days; error bars indicate s.d. of the total number and common superscripts above error bars indicate non-significant differences; *protozoal concentrations from strained rumen fluid through double layer of cheese cloth at slaughter on day 45.

Weight of gut tissue was the greatest for the rumen followed by the omasum and reticulum (Table 7.2). However, the total population of protozoa adhering to the wall of the rumen was not different from that adhering to the walls of the omasum. High numbers of entodiniomorphs were found in the omasum, but holotrichs were not detected.

Table 7.2 Ciliate protozoa in reticular, ruminal and omasal contents and adhering to the gut tissues of Angus heifers.

Parameter	Mean (n=8)			Pooled s.e	*P*-value
	Reticulum	Rumen	Omasum		
pH	6.29	6.38	6.28	0.13	0.83
Digesta weight in grams	905^{c}	34,113^{a}	4,540^{b}	678.3	<0.001
Total protozoa (×10^{6})	162.4^{b}	7,480.1^{a}	208.5^{b}	1.32	0.01
Large holotrichs	29.81^{b}	594.67^{a}	14.82^{b}	1.46	<0.001
Small holotrichs	60.40^{b}	775.11^{a}	15.67^{c}	1.44	<0.001
Large entodiniomorphs	19.43^{c}	1,966.5^{a}	46.81^{b}	1.28	<0.001
Small entodiniomorphs	37.71^{b}	3.677.5^{a}	111.05^{b}	1.45	<0.001
Gut tissue weight in grams	1,111.3^{c}	6,446.3^{a}	4,901.3^{b}	245.3	<0.001
Total protozoa (×10^{5})	0.65^{b}	3.31^{ab}	3.74^{a}	0.28	0.003
Large holotrichs	0.15^{b}	1.01^{a}	-	0.49	0.01
Small holotrichs	0.33^{b}	2.38^{a}	-	0.36	0.003
Large entodiniomorphs	0.12^{b}	1.62^{ab}	2.27^{a}	0.26	<0.001
Small entodiniomorphs	0.11^{b}	0.84^{ab}	2.31^{a}	0.28	<0.001

Different superscripts indicate significant difference within rows

7.4 Discussion

Rumen ciliate protozoa often represent approximately 1×10^{6} cells per mL in rumen contents (Dehority 2003), but protozoal concentrations vary among animals and are dependent on many factors such as ruminant species, geographical location (Akbar *et al.* 2009), diet (Whitelaw *et al.* 1984), frequency of feeding (Williams 1986) and rumen pH (Clarke 1977). Monitoring protozoal populations in rumen fluid in this study showed protozoal concentrations after 14 days of acclimatization period of changing to the diet of lucerne cereal hay mix were significantly higher than on the first day they started on the diet. The protozoal populations in rumen fluid after straining through a double layer

of cheese-cloth to remove large plant fibres were similar to that of samples collected from oesophageal intubation (Figure 7.1; $P > 0.05$), indicating that experiments involving sampling rumen fluid from oesophageal intubation for protozoal enumeration may offer an accurate estimate of protozoal populations being free in the liquid fraction of the rumen contents. This normal method of enumeration does not account for the protozoa adhering to plant particles (Bauchop and Clarke 1976) and these authors found high concentrations of rumen protozoa attaching on the damaged surface of plant fragments, between layers of plant cells and among vessel elements with the protozoa being identified as the entodiniomorphs: *Epidinium ecaudatum, Eudiplodinium spp., Diplodium spp.* and the holotrich *Dasytricha spp.* In addition, Czerkawski (1987) showed that protozoal populations in the rumen are divided into two distinct compartments: those freely suspended in the liquid phase, and those protozoa adhering to the fibrous mass of digesta. Hook *et al.* (2012) also reported the majority (63 - 90%) of rumen protozoa existed in the attached phase, either in the feed particles or in the rumen wall. Leng *et al.* (1981) who injected radioactivity-labelled protozoa in the rumen estimated between 60 and 90% of the total protozoa were available within the rumen to be sampled, but only 5 to 20% of the protozoal population was present in the fluid fraction.

The pH did not differ among reticular, ruminal and omasal contents and agreed with pH ranges found in previous studies (Prins *et al.* 1972; Towne and Nagaraja 1990). Rumen protozoa are significantly affected by the environment's acidity or alkalinity, with the protozoa unable to survive if rumen pH is above 7.8 or below 5.0 (Clarke 1977). Mackie *et al.* (1978) reported that protozoal numbers decreased by 50-80% if rumen pH

was below 5.4. The pH of ruminal or omasal contents in the present study were near neutral as the host animals were fed a roughage diet, meaning the omasum was therefore a suitable sequestration site for protozoa. Although the protozoal numbers in omasal contents were significantly fewer than in ruminal contents, which agreed with Weller and Pilgrim (1974) and Michalowski *et al.* (1986), similar numbers of protozoa were adhering to the ruminal and omasal walls.

Ciliate protozoa contribute disproportionately little to the nitrogen nutrition of ruminants with protozoal nitrogen being up to 53.4% of total microbial biomass in the rumen (Michałowski 1979) but only 20% of total microbial nitrogen entering to the duodenum (Jouany *et al.* 1988). The smaller protozoal biomass in the duodenum of the ruminants could reflect 65% - 74% of protozoa die and are degraded in the rumen of sheep or cattle, respectively (Leng 1982; Ffoulkes and Leng 1988), suggesting that only 24% - 35% of rumen protozoa enter to the lower digestive tract, while the majority of rumen protozoa are retained and lyse within the rumen. Further, the protozoal biomass leaving the rumen is greater than that arriving in the duodenum because of some rumen protozoa being trapped in the omasal leaves (Czerkawski 1987). The relatively high numbers of protozoa found in the omasum in this study may explain that the lower protozoal contribution to the total microbial nitrogen outflow in the duodenum is partly due to high numbers of protozoa being retained in the omasum of ruminants.

Elimination of rumen protozoa increases growth rate of ruminants (Bird and Leng 1978; Bird *et al.* 1979; Eugène *et al.* 2004a) especially when feed is deficient in protein rather than energy content. This leads to some circumstances in which rumen protozoa may

limit animal productivity. However, existing procedures to eliminate ciliate protozoa involve dosing ruminal contents with antiprotozoal detergents (Abou Akkada *et al.* 1968; Orpin 1977; Santra *et al.* 2007a; Bird *et al.* 2008; Hegarty *et al.* 2008), but these methods of defaunation are not always successful. In sheep, defaunation had been successfully with sodium 1-(2-sulfonatooxyethoxy) dodecane (Bird *et al.* 2008; Hegarty *et al.* 2008) or sodium lauryl sulfate (Santra *et al.* 2007a), but chemical defaunating detergents are not often reported successful in rendering cattle free of ciliate protozoa for prolonged periods (Bird and Leng 1978). Towne and Nagaraja (1990) completely eliminated rumen protozoa of Holstein steers by emptying ruminal contents, flushing the omasum and spraying 1 L of dioctyl sodium sulfosuccinate solution on rumino-reticulum walls and on the reticulo-omasal orifice. The authors found live protozoa in the rumen contents a day after the treatment.

The omasum, which is connecting the reticulorumen to the abomasum, transfers digesta from the reticulum into the abomasum (Van Soest 1994). The flow of digesta from the reticulum occurs following the omasal canal contractions, but occasionally backflow of large volumes of digesta from the omasum to the reticulum occurs when the omasal body contracts during the closure of omaso-abomasal orifice (Stevens *et al.* 1960). Therefore, Towne and Nagaraja (1990) claimed that the backflow of omasal contents containing residual of omasal protozoa re-inoculated the defaunated rumen of steers. That could explain why defaunation of cattle is difficult as residual omasal protozoa may be responsible for the reappearance of rumen protozoa after ruminal defaunation is complete.

7.5 Conclusion

The relatively large total populations of ciliate protozoa in the digesta and on the rumen wall of cattle indicate that the total populations of protozoa in the rumen cannot be simply counted by the conventional enumeration of fluid samples, although concentrations of rumen protozoa in the rumen fluid were similar in samples collected by oesophageal intubation and by slaughter. This study confirms there is a small but significant reservoir of protozoa in the omasum of cattle and it is proposed their backflow into the rumen contributes to reducing the effectiveness of chemical defaunation of the rumen. Techniques that completely eliminate protozoa from the omasum as well as the rumen of cattle will be required if the advantages of increasing the microbial nitrogen outflow and efficiency of feed utilisation associated with elimination of rumen protozoa are to be realised in the cattle industries.

Higher Degree Research Thesis by Publication

University of New England

Statement of Originality

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Chapter 8

General discussion

8.1 Introduction

Research on rumen protozoa has largely focused on their classification, effects on ruminal fermentation and protein yields (Williams and Coleman 1992), but as enteric CH_4 production from ruminants is the largest agricultural emission contributing to global warming, the role of rumen protozoa in moderating rumen methanogensis has received increasing attention (Newbold *et al.* 2015). Dietary strategies such as feeding of oils, saponins and NO_3 have also been examined to manipulate ruminal metabolism to reduce CH_4 production. Part of the action of some of these dietary interventions is through affecting rumen protozoal populations (Newbold *et al.* 2015), making it difficult to assess whether CH_4 mitigation is a direct effect of the additives or an indirect effect on protozoal density or both. Studies contrasting defaunated and refaunated animals, therefore, offer an opportunity to understand the contribution of protozoa to ruminal metabolism without confounding by diet ingredients, but even these have built an unclear understanding in the published literature of the role of protozoa (Williams and Coleman 1992). The major aim of this thesis was to understand the CH_4 production and animal productivity associated with defaunation and whether additive changes in CH_4 mitigation and productivity were possible by using defaunation and dietary additives together. The studies also sought to extend the applicability of defaunation by

measuring emissions of defaunated sheep while grazing and by investigating protozoal retention in the reticulum and omasum in order to facilitate future defaunation of cattle.

8.2 Protozoal impacts on the rumen and its fermentation and methane production

Perhaps the greatest consequence of defaunation for the rumen ecosystem and fermentation chemistry is a reduced predation of bacteria and an increased bacterial population. This increases microbial protein outflow and in turn increases animal productivity, especially where low protein diets are limiting animal production (Williams and Coleman 1992; Newbold *et al.* 2015). A lower concentration of NH_3 in the defaunated rumen was the most consistent effect of defaunation reported in this thesis and in the literature (Table 8.1). A decrease in rumen NH_3 level is a consequence of the absence of protozoa reducing both bacterial predation and the degradation of feed-protein in the rumen (Williams and Coleman 1992). The higher nitrogen flow into the duodeum results from an increase in feed nitrogen and microbial nitrogen flow (Ushida and Jouany 1990), leading to increased supply of amino acids to the host.

Table 8.1 Rumen metabolite concentration and methane production in the rumen fluid of defaunated animals normalized relative to those in faunated animals (1.00). Data are from experiments in this thesis and from published reviews.

	Total VFA	Acetate	Propionate	Butyrate	Acetate/ Propionate	NH_3	Methane production
Chapter 2	1.04	1.08*	0.89*	0.76*	1.21	0.74*	0.93*
Chapter 3†	0.91	0.96*	1.01	1.25*	0.94	0.73*	0.97
Chapter 4	0.84*	1.05*	0.85*	0.93	1.23*	0.66*	0.57*
Chapter 5‡	0.79	1.02	1.04	0.84	0.98	0.76*	0.62*
Chapter 6	1.16	1.01	1.10	0.89	0.91	0.61*	0.90*
Thesis mean	**0.95**	**1.02**	**0.98**	**0.93**	**1.05**	**0.70**	**0.80**
Published review							
Jouany *et al.* (1988)	0.94	0.98	1.32	0.94	0.90	0.76	
Hegarty (1999)		0.86	1.23	1.08	0.70		0.87
Eugène *et al.* (2004a)	0.96	0.98	1.14	0.87	0.86	0.70	
Newbold *et al.* (2015)	0.95	1.03	1.00	0.78	1.03	0.74	0.89

** Significant effect of defaunation ($P < 0.05$); † Experiment conducted under grazing environment; ‡ In vitro experiment.*

The extent of rumen fermentation and the balance of energy yielding substrates for the host are also important. The reduced total VFA concentration apparent in data averaged across studies in this thesis was consistent with the literature (Table 8.1). The lower total VFA concentration in the defaunated rumen could be due to a reduced rate of VFA production (Chapter 4) and/or a larger rumen volume in which the fermentation was occuring (Chapter 2). The experimental evidence presented in this thesis, however, confirms that protozoa effects on the molar proportions of VFA are not consistent. An increased molar proportion of propionate in the defaunated rumen was evident in many existing reviews (Jouany *et al.* 1988; Hegarty 1999; Eugène *et al.* 2004a), but our

results showed defaunation consistantly increased the molar proportion of acetate in agreement with Newbold *et al.* (2015) and subtantially reduced the molar proportion of butyrate, but did not significantly increase the molar proportion of propionate.

The results presented in this thesis support a conclusion that defaunation reduces CH_4 production both *in vitro* and *in vivo* (Table 8.1) and provided the first measurement of CH_4 production of defaunated ruminants while grazing. However, the percentage emission reduction was variable and mechanisms by which CH_4 emissions are reduced by defaunation are not clear. Hegarty (1999) proposed four possible mechanisms by which defaunation induces a lower CH_4 emissions, being; (1) reduced DM fermentation in the rumen (2) decreased endosymbiotic methanogens associated with rumen protozoa (3) modified ruminal VFA profile with increased molar proportion of propionate and decreased availability of H_2 (4) increased oxygen pressure in rumen fluid. Nevertheless, the experiments in this thesis recognise the complexity of the effect of defaunation on the ecosystem of the rumen, revealing a reduced CH_4 emissions can occur without an increased proportion of propionate.

By removing rumen protozoa, defaunation must eliminate the ecto- and endo-symbiotic habitats for physically associated methanogens (Finlay *et al.* 1994; Tokura *et al.* 1997; Kumar *et al.* 2013). However, as CH_4 emissions are not always decreased by defaunation (Kumar *et al.* 2013), alternative methanogen populations may arise and replace those of the protozoa-associated methanogens (Morgavi *et al.* 2012). The changes in the methanogenic community following defaunation are inconsistent among studies (McAllister and Newbold 2008; Mosoni *et al.* 2011; Morgavi *et al.* 2012; Kumar

et al. 2013). Reduced CH_4 emissions following defaunation in these studies may be due to reducing the most active CH_4 methanogens in the rumen and the substitution of other methanogenic populations which are less able to utilise H_2 to produce CH_4. Another possibility is that in the absence of protozoa other populations of microbes establish or increase in the rumen that may enable alternative sinks for H_2 that have a higher affinity for H_2 than do methanogens. While our circumstantial data supports this with more acetate and less CH_4 as may be expected if reductive acetogenesis was active (Chapter 2, 4 and 6), this hypothesis was not tested and needs future investigation as it is recognised that reductive acetogenesis is not a normal ruminal reaction.

The results from Chapter 2 showed that the absence of rumen protozoa tended to increase reticulo-rumen weight and significantly increase the ratio of reticulo-rumen to whole body weight. The increased weight of rumen contents are often seen after defaunation due to a longer particle retention of rumen digesta (Chapter 4) associated with the rumen fill effect of reducing OM (Eugène *et al.* 2004a) and/or whole tract DM digestibility (Chapter 4). This physical change in the rumen size following defaunation, however, is not always observed (Williams and Coleman 1992).

Although the changes in rumen characteristics and VFA molar proportions are not always consistent following defaunation, this thesis has clearly demonstrated the consistent influence of rumen protozoa on NH_3 concentration. The reduced ruminal catabolism of engulfed feed-protein and bacteria as well as reduced CH_4 emissions are advantages of defaunation. These advantages would favour an enhanced efficiency of

nutrient utilisation and production of ruminants, especially those with a high amino acid requirement and/or when consuming feeds of very low protein content.

8.3 Growth and productivity of defaunated ruminants

The demand for products from livestock, especially in the tropics where forage is often deficient in protein content, is increasing. The requirement to produce at least 70% more food in order to feed 9 billion people by 2050 (World Bank 2008) is a major challenge to animal production. The world livestock population has surged in many developing countries in response to this rapid growing demand for livestock products (FAO 2006). In Asia, the majority of ruminants are fed protein deficient diets from locally produced and available by-products due to an increasing competition for feed between human consumption and monogastric livestock demands (Devendra and Leng 2011).

Defaunation of the rumen offers an opportunity to optimise productivity of ruminants in such protein-scarce environments. This thesis demonstrated a consistent effect of defaunation to increase microbial protein outflow which increased protein supply to the host for liveweight gain (Table 8.2). Although a positive response to defaunation in animal performance is not always significant in the literature, there are no apparent negative effects. A small decrease in DM digestibility due to defaunation in this thesis and in the review was not associated with reduced animal growth, suggesting that greater microbial protein supply may lead to higher feed conversion efficiency in defaunated ruminants. Indeed, defaunation has increased feed conversion efficiency due to a greater efficiency of nutrient utilisation for absorption compared to conventional animals (Newbold *et al.* 2015). These positive effects of defaunation on animal growth

are often seen with poor quality roughage diets that are low in fermentable carbohydrate and rumen degradable nitrogen for the growth of rumen microbes (Chapter 4).

Table 8.2 Dry matter intake, digestibility, microbial protein outflow, liveweight gain and wool growth of defaunated ruminants normalized relative to those of faunated ruminants (1.00). Data are from experiments in this thesis and from published reviews.

	DM intake	DM digestibility	Microbial protein outflow	Liveweight gain	Wool growth	Wool fibre diameter	Greasy fleece weight
Chapter 2	0.98*	0.97	1.17	1.04			
Chapter 3†	1.05			1.03	0.97	1.03	1.02
Chapter 4	0.94	0.94*	1.03	1.11	0.95	1.01	
Chapter 6	0.98	1.03	1.29*	1.56			
Thesis mean	**0.99**	**0.98**	**1.16**	**1.18**	**0.94**	**1.02**	**1.02**
Published review							
Jouany *et al.* (1988)	1.04	0.94	1.56	1.02			
Eugène *et al.* (2004a)	1.01	0.98	1.12	1.11	1.14		
Newbold *et al.* (2015)	0.98	0.96	1.30	1.09	1.01		

** Significant effect of the defaunation ($P < 0.05$); † Experiment conducted under grazing environment*

The experiments reported in Chapter 3 and 4 showed that wool production was not positively increased by defaunation, but wool fibre diameter was slightly increased (Table 8.2). Previous studies have shown positive effects of defaunation on wool growth (Eugène *et al.* 2004a) and longer experimental periods or higher statistical power in the current experiments may have likely shown similar results. This thesis also showed a greater liveweight gain response in defaunated cattle (Chapter 6) associated with a greater quantity of microbial protein which flows from the bovine rumen is available for muscle growth. This suggests that defaunation of beef cattle would have

both commercial and environmental benefits, and for these reasons, the study of protozoa in the forestomach of cattle was undertaken (Chapter 7; section 8.5).

8.4 Defaunation and dietary oil or nitrate as complementary mitigation strategies

Many of the leading feed additives showing efficacy in CH_4 mitigation are likely to be commercially constrained due to either toxicity risk (NO_3; Leng and Preston (2010)) or suppression of DM intake and DM digestibility (eg. oils; Patra (2013)), that limits their inclusion in the diet. Recently, Hristov *et al.* (2015) reported an active compound of 3-nitrooxypropanol (3NOP) that persistently reduces CH_4 emissions in dairy cattle. Thus, there is interest in combining mitigation strategies to achieve greater total CH_4 mitigation. Our studies, like those of Guyader *et al.* (2015) and Troy *et al.* (2015) combined defaunation with supplements of dietary NO_3 (Chapter 4) and coconut oil (Chapter 6) to supplement animals with NPN (NO_3) or energy (coconut oil) which may be both advantageous to animal eating low quality forage. Results from Chapter 4 showed that supplementation with NO_3 increased DM intake and DM digestibility, rumen fermentation and ADG while supplementation of coconut oil reported in Chapter 6 did not improve rumen fermentation or animal productivity, largely as a result of suppressing DM intake.

Although supplementation of NO_3 or coconut oil independently reduced CH_4 production, combining these with defaunation resulted in different outcomes for CH_4 mitigation. The results presented in Chapter 4 showed for the first time that defaunation and NO_3 positively interacted to reduce CH_4 yield and that NO_3 in combination with

defaunation additively increased DM intake and decreased CH_4 production. Nitrate is known to serve as a H_2 sink that could favour NH_3 formation over CH_4 formation in the rumen, and it also indirectly decreases the methanogen population (van Zijderveld *et al.* 2011). Combining defaunation with NO_3 could be further affecting methanogens to reduce CH_4 production in an additive manner compared to NO_3 treatment alone.

The lack of interaction between defaunation and coconut oil reported in Chapter 6 supported the hypothesis that both treatments would additively deliver greater mitigation of CH_4 emissions compared to defaunation or oil treatment alone. Combination of defaunation and coconut oil showed the reduced rumen NH_3 concentration and microbial protein outflow from defaunated rumen. This is because coconut oil not only suppresses the rumen protozoal population which indirectly decreases CH_4 production, but also suppresses the rumen bacteria (Dohme *et al.* 1999), which constrains microbial synthesis in the rumen. The non-significant interaction between defaunation and coconut oil on CH_4 mitigation reported in Chapter 6 confirms a previous study by Machmüller *et al.* (2003) who found no interaction for methane production, but a higher density of methanogens in the rumen fluid of defaunated sheep when fed coconut oil. Therefore, CH_4 mitigation was not found to be further enhanced by supplementing defaunated animals with oils.

Studies from this thesis confirm that supplementation with NO_3 as a NPN source (but not coconut oil) enhanced defaunated animal productivity and additively reduced CH_4 emissions.

8.5 Challenges to applying defaunation in commercial practice

Despite the fact that elimination of rumen protozoa has shown potentially positive impacts on improving animal productivity and reducing enteric CH_4 emissions from ruminants, there are no defaunation methods that are safe, effective and practically applicable for commercial enterprises. For research purposes, Jouany *et al.* (1988) had reviewed different techniques used to defaunate the rumen. However, these techniques are not always successful in rendering ruminants, especially cattle, free from protozoa for a prolonged period of time.

This thesis further advances the technique of Bird and Light (2013) to defaunate the ovine rumen by feeding animals with coconut oil distillate rich in lauric acid to suppress rumen protozoa for at least 7 days before three days of orally dosing with sodium 1-(2-sulfonatooxyethoxy) dodecane. However, the dosing procedure may still require repeating for permanent defaunation. This defaunation technique suppresses animal intake for an average of 10 days, but it is still not clear what period of time is required for the microbial ecosystem to fully stabilise.

Defaunation of cattle in this study was not easy achieved as entodiniomorph protozoa were visually observed 4 weeks after the experiment finished, so the cattle were visually free from ciliate protozoa for only 12 weeks. Defaunation of cattle, therefore, is more difficult than for sheep and this is thought to be attributable to different anatomical structures between the ovine and bovine rumen and omasum that restrict the exposure of protozoa to the defaunating chemicals (Towne and Nagaraja 1990). The finding of a small but significant protozoal population in the omasum (contents and adhering to the

wall, Chapter 7) supports the hypothesis of Towne and Nagaraja (1990) that protozoa residing in the omasum could provide a reservoir of organisms that re-infect the rumen after defaunation treatments have ceased. This presents a commercial challenge to use of oral or in-feed defaunating treatments and suggests an antiprotozoal compound that is carried in blood before diffusing into the gastrointestinal tract may be required to successfully defaunate cattle commercially. Since no such compounds are available, this is a major constraint to defaunation of bovines in the tropical regions where protein deficiency is limiting productivity of ruminants.

8.6 Conclusion

Studies in this thesis confirm for the first time that sheep without protozoa have a lower CH_4 yield while grazing than do faunated sheep, although the mitigation is moderate and only statistically different with $P < 0.1$. Across all housed animal and grazing studies the effects on rumen fermentation were mixed, as evident in existing literature. Consequently, defaunation cannot be relied upon to change fermentation patterns in a predictable way. The reliability of changes in ruminal and animal nitrogen metabolism in the thesis and wider literature are, however, consistent and of sufficient importance to motivate defaunation of developing anti-protozoal treatments for application in the tropics where low protein diets are common. It is suggested complete defaunation of cattle (and presumably buffalo) will require a compound that contacts protozoa by both direct contact in digesta and by diffusion from the blood to eliminate protozoa sequestered in the omasum.

To provide assurance of CH_4 mitigation and enhanced animal productivity it is recommended that defaunation be combined with other mitigation strategies such as NO_3 feeding where additive effects from NO_3 can be expected to not only further reduce emissions, but also improve animal performance and potentially improve reproductive outcomes due to improved body weight or condition score of tropical ruminants.

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