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Nitrate and nitrite metabolism in ruminant livestock

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Doctor of Philosophy

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Certification of dissertation

I certify that the ideas, experimental work, results, analyses, software and conclusions reported in this dissertation are entirely my own effort, except where otherwise acknowledged.

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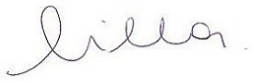

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Executive summary

Global methane (CH_4) emissions from ruminant livestock have continued to increase in the last decade due to the global ruminant population increasing in response to growing demand for livestock products. Supplementing the diet of ruminants with nitrate (NO_3) is an effective strategy to reduce enteric CH_4 emissions and also provides additional non-protein nitrogen for the growth of rumen microorganisms. However, there is a risk of nitrite (NO_2) toxicity in ruminants after feeding NO_3 that is unpredictable because of the large between-animal variation in NO_3 metabolism. The main objective of this thesis was to provide new knowledge about the basic biology of NO_3 absorption and NO_2 formation in ruminants, in order to increase the safety of NO_3 supplementation. This thesis includes a review of the literature and five experimental chapters where NO_3 and NO_2 metabolism and the effects of dietary NO_3 on CH_4 emissions, together with ruminants and the physiological effects of dietary NO_3 on insulin sensitivity in sheep, were evaluated. The results obtained from this thesis indicate:

- Feeding dietary NO_3 in combination with canola oil has a more than additive effect on reducing methanogenesis in cattle.
- Dietary NO_3 provided to sheep fed an otherwise highly protein-deficient diet reduces CH_4 emissions and provides additional N for microbial growth, including through NO_3-N recycling via saliva into the gastrointestinal tract.
- Large between-animal variability in NO_3 metabolism *in vivo* and *in vitro* was observed throughout this thesis.
- The presence of protozoa in the rumen of sheep adapted to dietary NO_3 increases NO_3 reduction to NH_3 without stimulating accumulation of NO_2 in the rumen; reducing the risk of NO_2 toxicity in ruminants.
- Faecal and urinary recovery of an intravenous dose of $^{15}NO_3$ was 64% after 6 days. Urinary urea-N was labelled confirming the passage of plasma NO_3 to the digestive tract of sheep had occurred.
- This thesis found evidence of NO_3 accumulating in tissues of sheep for at least one hour after intravenous injection of $^{15}NO_3$ and $^{15}NO_2$.
- Evidence of NO_3 and NO_2 being rapidly absorbed from the rumen of sheep into the blood was found. Once in the bloodstream, NO_2 was oxidised to NO_3 which is recycled via enterosalivary circulation in like manner to urea.

- Nitrate and NO_2 are highly absorbed from the small intestine into the bloodstream, with NO_2 absorption responsible for high levels of blood methaemoglobin. Absorption of NO_3 from the abomasum to the blood was evident, whereas it is hypothesised that NO_2 was converted to nitric oxide in the acidic conditions of the abomasum so did not lead to high blood NO_2.
- Nitrate is highly concentrated in saliva relative to blood NO_3 concentration.
- We found no evidence that feeding a low dose of dietary NO_3 (18 g NO_3/kg DM) affects insulin sensitivity in sheep.

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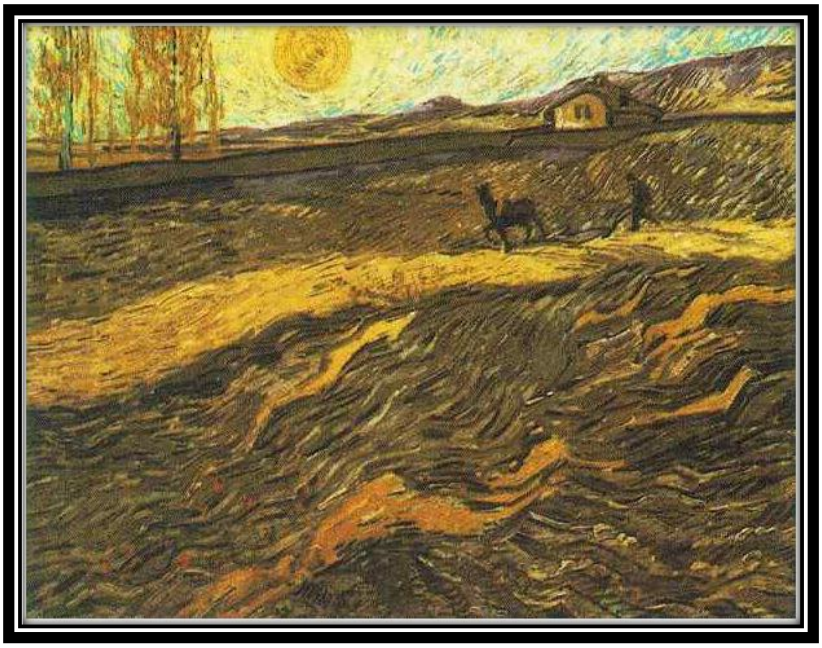
List of abbreviations

ADG	Average Daily Gain
AIRg	Acute Insulin Response
BW	Body Weight
BWG	Body Weight Gain
CH_4	Methane
CO_2	Carbon dioxide
Co-EDTA	Cobalt Ethylenediaminetetraacetic acid
CP	Crude Protein
Cr-NDF	Neutral Detergent Fibre mordanted with chromium
CSH	Cysteamine Hydrochloride
DEF	Defaunated
DI	Disposition index
DM	Dry Matter
DMADR	Dry Matter as Digested in the Rumen
DMD	Dry Matter Digestibility
DMI	Dry Matter Intake
DMP	Daily Methane Production
DOMI	Digestible organic matter intake
EN	Encapsulated Nitrate
FAU	Faunated
FP	Fluid Phase
G_b	Basal plasma glucose
GE	Gross Energy
GEI	Gross Energy Intake
GHG	Greenhouse Gas
GIT	Gastrointestinal Tract
GLUT4	Glucose Transporter Type 4
H_2	Hydrogen
Hb	Haemoglobin
I_b	Basal plasma insulin
ILR	Irreversible Loss Rate
IQR	Interquartile Range
LW	Liveweight
ME	Metabolize Energy
MetHb	Methaemoglobin
MINMOD	Minimal Model
MicNAN	Microbial non-ammonia Nitrogen
MRT	Mean Retention Time
MY	Methane Yield
N	Nitrogen
N_2	Dinitrogen

N_2O	Nitrous Oxide
NAN	Non-Ammonia Nitrogen
NFD	Neutral Detergent Fibre
NH_3	Ammonia
NO	Nitric Oxide
NO_2	Nitrite
NO_3	Nitrate
NOS	Nitric Oxide Synthase
NPN	Non-protein Nitrogen
OM	Organic Matter
OMDR	Organic Matter Digested in the Rumen
P	Protozoa
PP	Particulate Phase
RDF	Rumen Digesta Fraction
S_I	Insulin sensitivity
S_G	Glucose efectiveness
TD	True Digesta
Ur	Urea
VFA	Volatile Fatty Acids
WRD	Whole Rumen Digesta

Chapter 1. Literature review



Literature review

1.1. Use of nitrate as a dietary additive for reducing enteric methane production

1.1.1. Contribution of enteric methane to greenhouse gases emissions

Methane (CH_4) produced by livestock accounts for 6-7% of total global anthropogenic carbon dioxide equivalent emissions and ~35% of total anthropogenic CH_4 emissions (Reay, Smith, Christensen, James & Clark, 2018). Life cycle analysis shows CH_4 accounts for 43% of agricultural emissions, and the remaining part is almost equally shared between nitrous oxide (N_2O; 29%) and carbon dioxide (CO_2; 27%) (Herrero et al., 2016). Mitigation of CH_4 emissions is possible through various strategies such as use of feed additives, improving feed digestibility, manure management, increased animal productivity and health, plus grazing-land management practices (Herrero et al., 2016). At present, the feeding management approaches are the most developed and ready to be applied in the field (Martin, Morgavi & Doreau, 2010). The challenge is to reduce CH_4 production from ruminants without lowering animal productivity, so improving efficiency of use of dietary energy. The nutritional means of mitigating emissions have been widely reviewed (Martin et al., 2010; Grainger & Beauchemin, 2011; Patra, 2012; Patra, 2013) and the following review assesses the underlying science and practical evidence of dietary nitrate (NO_3) as an emerging mitigation tool for enteric CH_4.

1.1.2. Methane production in the rumen

Fermentation of feed components by the rumen microbiota under anaerobic conditions, results in the liberation of volatile fatty acids (VFA; mainly acetate, propionate and butyrate) as energy sources for the animal and gases (CO_2, H_2: hydrogen and CH_4) (Figure 1.1). Over 90% of the enteric CH_4 has its origin in the rumen and is mainly eliminated through eructation (Murray, Bryant & Leng, 1976). Enteric CH_4 released to the atmosphere is an inefficiency of livestock production and represents a loss between 2 to 12% of gross energy intake (Blaxter & Czerkawski, 1966; Johnson & Johnson, 1995).

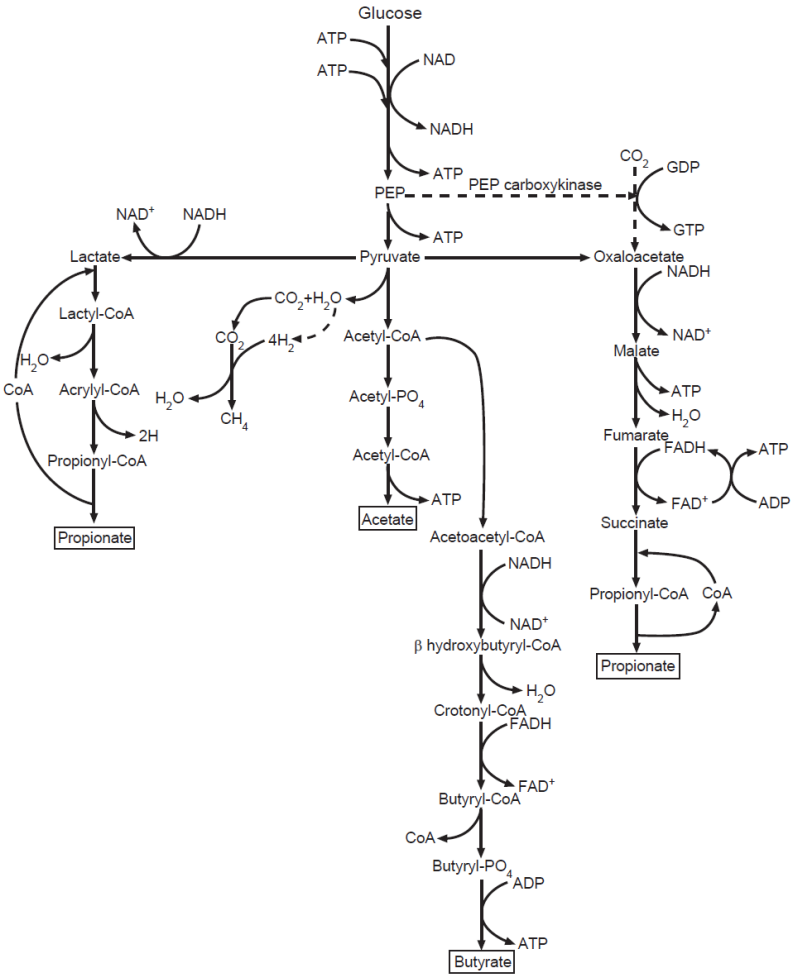


Figure 1.1. Principal fermentation reactions in the rumen that generate or utilise hydrogen (Hegarty, 1999).

Rumen fermentation is a series of oxidation and reduction reactions in which high energy electrons are transferred to reduce cofactors which are in turn used in production of H_2 (Table 1.1) which is ultimately used by methanogenic bacteria to reduce CO_2 to CH_4. This avoids H_2 accumulation and keeps the partial pressure of H_2 at the correct levels for normal rumen function (Moss, Jouany & Newbold, 2000; Martin et al., 2010). Because the rate of rumen CH_4 production is positively associated with the concentration of H_2 dissolved in the rumen (Czerkawski, Harfoot & Breckenridge, 1972; Hegarty, 1999), strategies to mitigate CH_4 emissions by ruminants should consider H_2 production in the rumen and its use by the methanogenic archaea.

Table 1.1. Volatile fatty acid production and reductive processes in the rumen (Kohn & Boston, 2000; Ungerfeld, 2013; Haque, 2018)

Substrate	Products	Reactions
VFA Production		
$C_6H_{12}O_6 + 2H_2O$	$2C_2H_4O_2 + 2CO_2 + 8H^+$	Acetate production
$C_6H_{12}O_6 + 4H^+$	$2C_3C_6O_3 + 2H_2O$	Propionate production
$C_6H_{12}O_6$	$C_4H_8O_4 + 2CO_2 + 4H^+$	Butyrate production
Other reductive process		
$CO_2 + 4H_2$	$CH_4 + 2H_2O$	Methane production
$2CO_2 + 4H_2$	$C_2H_4O_2 + 2H_2O$	Reductive acetogenesis
$SO_4^{2-} + 4H_2 + H^+$	$HS^- + 4H_2O$	Sulfate reduction
$NO_3^- + 4H_2 + 2H^+$	$NH_4 + 3H_2O$	Nitrate reduction

1.1.3. Effect of nitrate on methanogenesis

Highly oxidised inorganic compounds such as NO_3 salts (and sulphate) have been proposed as alternative H_2 sinks and so electron consumers, competing with methanogenesis for H_2 available in the rumen (Czerkawski, 1986; Leng, 2008; Latham, Anderson, Pinchak & Nisbet, 2016). Rumen microbes use H_2 to reduce NO_3 to nitrite (NO_2) and finally to ammonia (NH_3); meaning any electrons used in NO_3 reduction are not available for CH_4 formation (Lewis, 1951a; Jones, 1972). One strategy to mitigate CH_4 production by ruminants is shifting the fermentation balance from acetate and H_2 production to propionate fermentation which is a net proton consumer (Hegarty, 1999). Additionally, the reduction of NO_3 is thermodynamically more favourable (ΔG^o = -598kJ; (Allison & Reddy, 1984) than the reduction of CO_2 to CH_4 (ΔG^o= -175kJ; Conrad and Wetter 1990) and NO_3 has higher affinity for H_2 than the reactions that generate propionate (Kristjansson, Schönheit & Thauer, 1982; Ungerfeld & Kohn, 2006). Therefore, when NO_3 is added to the diet of ruminants a reduction in propionate production is expected (Farra & Satter, 1971). Conversely, once the amounts of NO_3 dosed exceeds the capacity of rumen microbes to reduce it to NH_3, direct inhibition of methanogens by NO_3 or NO_2 might occur (van Zijderveld et al., 2010; Lee & Beauchemin, 2014). The mechanism of action of NO_3 on rumen fermentation beyond its direct role as a H_2 sink requires further study (Guyader, Tavendale, Martin & Muetzel, 2016; Yang, Rooke, Cabeza & Wallace, 2016).

Stoichiometrically, the same amount of H_2 is utilised to reduce 1 mole of NO_3 as 1 mole of CO_2, so 1 mole of NO_3 (62 g/mol) should reduce 1 mole of CH_4, equivalent to 16 g or 22.4 L if other H_2 transactions were unaffected (Nolan, Godwin, de Raphélis-Soissan & Hegarty,

2016). A meta-analysis reported that 1% NO_3 added to the diet of cattle mitigated CH_4 emissions by 10% on average (Lee & Beauchemin, 2014). Methane mitigation by feeding NO_3 has been extensively studied *in vivo* with findings summarised in Table 1.2. However, because of NO_3 and NO_2 excretion in urine, changes in VFA proportions produced and changes in rumen microbiota associated with H_2 production, the efficacy of CH_4 mitigation effect from feeding NO_3 is not rarely at the theoretical level. As the amount of NO_3 inclusion increases the efficiency of NO_3 mitigation decreases (Leng, 2014) as has been found *in vitro* and some reduction in DMI has been observed (Table 1.2).

Table 1.2. Summary of *in vivo* results after supplementing dietary nitrate (NO_3) during diverse periods and at different levels on methane yield (MY; g CH_4/kg DMI), dry matter intake (DMI), dry matter digestibility (DMD) and animal performances (body weight gain, BWG (kg/d); milk yield (L/d) or wool growth, µg/cm^2/d) evaluated in ruminant livestock. (EN: encapsulated nitrate).

Animal	NO_3 (g/kg DM)	Days	CH_4 yield	DMI	DMD	Performance	Reference
Dairy	14.6	70	-15%	na	ns	+4% milk	Wang et al. (2018)
Beef	57.28*	31	ns	ns	ns	ns	Goopy and Hegarty (2018)
Dairy	11- 23	78	-4,-10%	ns	na	-11% milk	Van Wyngaard et al. (2018)
Beef	21	56	-8%	ns	na	-11% BWG	Duthie et al. (2018)
Beef	12.5-25 (EN)	28	ns	-8%	na	+0.9% BWG	Lee et al. (2017a)
Beef	20 (EN)	91	ns	ns	na	BWG: ns	Lee et al. (2017b)
Sheep	5.1 (EN)	9	-13%	ns	na	na	de Raphélis-Soissan et al. (2017)
Beef	9.1-18.8	88	na	-9%	na	-2% BWG	Hegarty et al. (2016)
Dairy	5.3, 13.6, 21.1	140	-6, -13,-23%	ns	ns	Milk: ns	Olijhoek et al. (2016)
Lambs	19	93	ns	+1.5%	+1.1%	+100% BWG	Nguyen et al. (2016)
Dairy	21	17	-17.5%	-5%	ns	Milk: ns	Klop et al. (2016)
Beef	4.6- 7.9	112	-15%	+10-14%	ns	na	Tomkins et al. (2016)
Beef	18	56	na	ns	na	BWG: ns	Duthie et al. (2016)
Dairy	22	70	-22%	ns	ns	na	Guyader et al. (2015)
Dairy	20	40	-14% -18%	-8%	na	-18% milk	Veneman et al. (2015)
Beef	11-48 (EN)	28	na	-7.7	na	na	Lee et al. (2015a)
Beef	10, 20 or 30 (EN)	112	na	-3%	na	na	Lee et al. (2015a)
Beef	1.5-25 (EN)	112	-4.2-18%	-3%	+3%	na	Lee et al. (2015b)
Sheep	20	70	-15%	-0.8%	na	+12% wool	de Raphélis-Soissan et al. (2014)
Lambs	27.4 (EN)	92	-33%	ns	na	BWG: ns	El-Zaiat et al. (2014)

Animal	NO_3 (g/kg DM)	Days	CH_4 yield	DMI	DMD	Performance	Reference
Dairy	20	4	-25	na	na	na	Lund et al. (2014)
Beef	6 to 30	33	-12% to -29%	na	na	BWG: ns	Newbold et al. (2014)
Beef	6 to 24	111	na	-3.2%	na	na	Newbold et al. (2014)
Beef	26	28	+0.06%	ns	na	BWG: ns	Velazco et al. 2014)
Lambs	18.8	69	-24%	ns	ns	+37% wool	Li et al. (2013)
Beef	22	46	-27%	ns	na	na	Hulshof et al. (2012)
Sheep	19	54	-35.4%	ns	ns	BWG: ns	Li et al. (2012)
Dairy	21	96	-16%	na	na	Milk: ns	Van Zijderveld et al. (2011)
Sheep	25	21	-23%	ns	ns	na	Nolan et al. (2010)
Sheep	26	35	-32%	ns	na	BWG: ns	Van Zijderveld et al. (2010)

*included in molasses-based liquid supplement

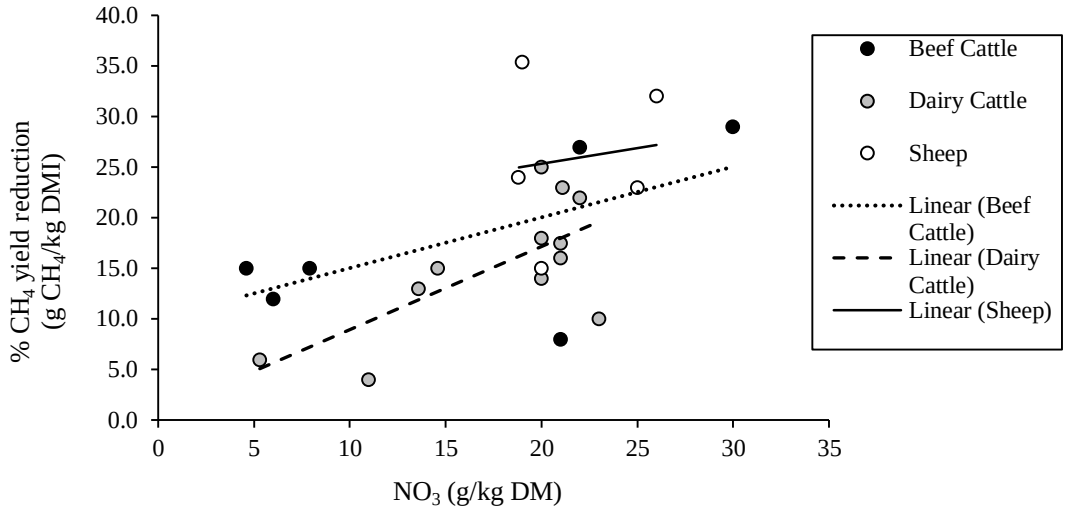


Figure 1.2. Relationship between methane yield reduction (MY; g CH_4/kg DMI) as a proportion of dry matter intake (DMI) and the dose of nitrate added to the diet of beef cattle, dairy cattle and sheep.

Figure 1.2 includes those experiments from Table 1.2 with significant effects on reducing methane yield (MY; g CH_4/kg DMI) and excludes experiments with encapsulated nitrate (EN). Across all species, there is a positive relationship between the effectiveness of NO_3 on reducing MY and the increasing dose of NO_3 added to the diet of ruminants. Doses with more than 19 g NO_3/kg DM in the diet increased the variability on MY reduction relative to lower doses.

1.1.4. Nitrate as a valuable source of non-protein nitrogen

In addition to interest in dietary NO_3 as a means to mitigate enteric CH_4 emissions, it may also offer advantage as a non-protein nitrogen (NPN) source to stimulate the rate of rumen microbial fermentation. The practice of supplementing ruminants with an NPN source is common for low quality forage diets. Intakes of 23-46 g urea/animal/day in cattle have shown liveweight (LW) responses of 0.1 to 0.25 kg/day in cattle on tropical forages (Coates & Dixon, 2008) and supplementation with urea is one of the few strategies available to the extensive beef industry to reduce mortality and LW losses during the dry season (Callaghan, Tomkins, Benu & Parker, 2014). Supplementing ruminants with NO_3 can replace urea as source of NPN while also reducing enteric methanogenesis (Leng, 2008; Nolan, Hegarty, Hegarty, Godwin & Woodgate, 2010; Van Zijderveld et al., 2011), so the value of the extra performance can offset the cost of NO_3. Similar improvements in animal performance have been reported when either NO_3 or urea were included in the diet of ruminants at similar levels (Lee & Beauchemin, 2014).

However, this effect has not always been observed. Hegarty et al. (2016) reported that increasing NPN inclusion (as NO_3 or urea) from 0.25 to 0.45% NPN in dietary DM and replacing urea with calcium NO_3 in a protein-rich feedlot diet decreased LW gain in cattle without improving feed efficiency.

Most of the ingested NO_3 is finally reduced to NH_3 in the rumen which is the main N source for microbial growth. The inclusion of supplementary NO_3 in the diet of ruminants usually becomes less appropriate when the crude protein content of the basal feed DM exceeds 10% or when the metabolize energy (ME) content of the feed and so N requirement is low (Nolan et al., 2016). Additionally, it was reported that NO_3 inclusion in high concentrate diets was less effective in mitigating CH_4 emissions due to fundamental differences in NO_3 metabolism between concentrate and mixed (forage and concentrate) diets (Troy et al., 2015). Only by better understanding the ruminal and animal metabolism of NO_3 and its metabolites can the safety and efficacy of dietary NO_3 as a tool to stimulate fermentation and inhibit methanogenesis be defined.

1.2. Fundamental biology of nitrate metabolism in ruminants

1.2.1. Nitrate and nitrite metabolism in the rumen

1.2.1.1. Nitrate and nitrite reduction in the rumen

Nitrate can occur in drinking water and is a common component of crude protein in forages (Dawson, Rasmussen & Allison, 1997). It is also present in some commercial fertilizers; so it may be consumed by grazing ruminants on a daily basis. Toxic levels of NO_3 in feed are usually associated with excessive levels of dietary crude protein leading to high NH_3 concentrations in the rumen (Leng, 2008). However, at lower inclusion levels, most ingested NO_3 is converted to NH_3 in the rumen, providing N for microbial growth (Lewis, 1951a; Wang, Garcia-Rivera & Burris, 1961). Some bacteria present in the rumen microbiome are equipped with highly efficient NO_3^- reductase enzymes and use NO_3 for respiration or as a substrate for incorporation of nitrogen into biomass (Lundberg & Govoni, 2004).

When NO_3 enters into the rumen, it cannot be used as an N source directly; it must be first reduced to NO_2 and then to NH_3 by rumen microorganisms. Microbial reduction of NO_3 can occur by dissimilatory or assimilatory processes. The reduction of NO_3 to produce rumen NH_3 (dissimilatory nitrate reduction; Figure 1.3) seems to be the major cause of the initial rapid rate of disappearance of NO_3 (Leng, 2008). Nitrate is also reduced to NO_2 (dissimilatory nitrate reduction; Figure 1.3), as an intermediate product. Rumen microbes use the electrons from the H_2 to reduce NO_3 and NO_2 according to equations 1 and 2 (Nolan et al., 2016):

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \quad (1)$$

$$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^- + 2H_2O \quad (2)$$

Nitrate can also be reduced to nitrous oxide (N_2O) and further to dinitrogen (N_2) via denitrification, serving as a metabolic route for electron disposal to the reduction of NO_3 (Figure 1.3). However, the reduction of NO_3 to NH_3 is thermodynamically more favourable than the reduction of NO_2 to N_2O ($-\Delta G = 436$ kJ/mole vs. $-\Delta G = 453$ kJ/mole), and thus NH_3 is the prevalent reduction product in the rumen (Latham et al., 2016). The maximum NO_3 reduction occurs when rumen pH is 6.5 and NO_2 reduction is maximal under more acidic conditions at pH 5.6 (Geurink, Malestein, Kemp & van't Klooster, 1979; Johnson, Schneider, Kelling & Doster, 1983).

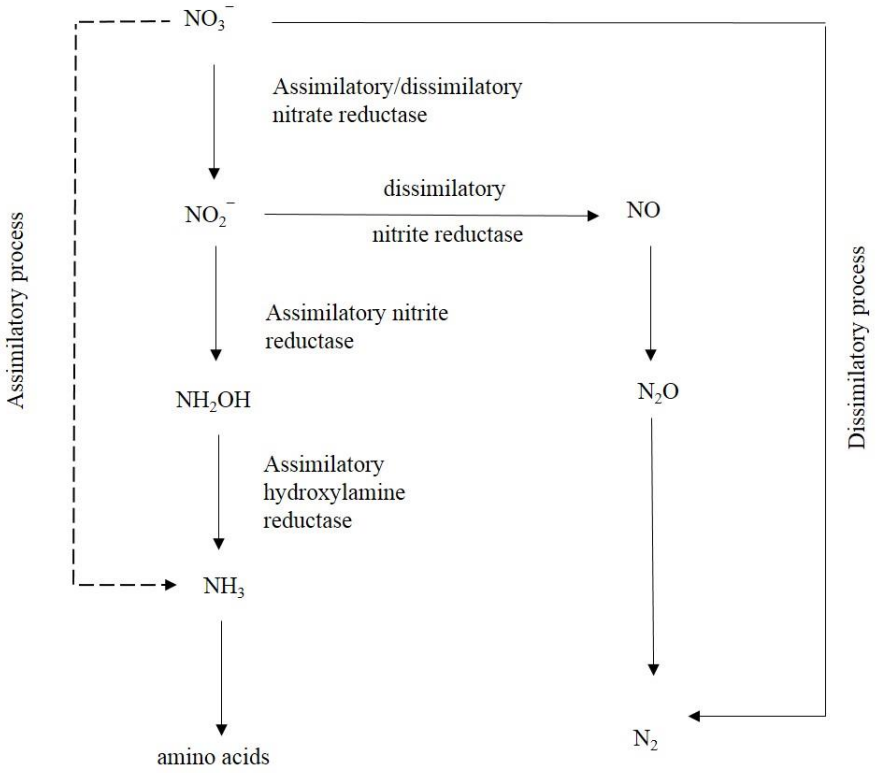


Figure 1.3. Nitrate reduction pathways in the rumen. Assimilatory and dissimilatory routes of nitrate (NO_3) and nitrite (NO_2) reduction. Adapted from (Yang et al., 2016)

1.2.1.2. The assimilatory and dissimilatory pathway of nitrate and nitrate metabolism

Assimilatory and dissimilatory reductases are present in the rumen biota (Jones, 1972; Kaspar & Tiedje, 1981) and the activity of both enzymes appear to increase during an adaptation period to NO_3 (Allison & Reddy, 1984; Alaboudi & Jones, 1985; Asanuma, Yokoyama & Hino, 2015). Microbial reduction of NO_3 can occur by dissimilatory or assimilatory processes (Figure 1.3) and the genes involved in both pathways differ substantially (Latham et al., 2016).

Nitrate reduction via the assimilatory pathway requires energy to reduce NO_3 to NH_3 as a source for microbial protein synthesis and is repressed by NH_3 (Moreno-Vivián, Cabello, Martínez-Luque, Blasco & Castillo, 1999). Therefore, considering that the rumen is an environment rich in NH_3 the functional role of this process seems to be minimal since NH_3 concentration may down-regulate the activity of this process (Latham et al., 2016). Dissimilatory NO_3 reduction to NH_3, contrarily, is a process that generates energy carried out by obligate and facultative anaerobic bacteria (Thauer, Jungermann & Decker, 1977) and in the rumen takes place in a two-step pathway. Firstly, NO_3 is reduced to NO_2 and further to

NH_3. Normally, the reduction of NO_2 to NH_3 is much slower than the reduction of NO_3 to NO_2 (Dawson et al., 1997) and depending on the balance of the enzymes, intermediates such as NO_2, nitric oxide (NO) or N_2O can occur at any step (Yang et al., 2016). When NO_3 or NO_2 accumulate in the rumen they affect physiological processes in the animal and in particular, high concentrations of NO_2 inhibit the growth of rumen microorganisms (Marais, Therion, Mackie, Kistner & Dennison, 1988; Bruning-Fann & Kaneene, 1993).

Enzymes involved in dissimilatory NO_3 reduction include membrane bound and periplasmic NO_3 reductases encoded by *nar* and *nap* genes and NO_2 reductases encoded by *nir* and *nrf* genes (Thauer et al., 1977; Moreno-Vivián et al., 1999; Latham et al., 2016). Dissimilatory NO_2 reduction occurs on the outer cytoplasmic membrane and depending on the organism, receives electrons via carriers such as NADH or FADH and upon subsequent transfer of these electrons to the respiratory electron transport system they can be used to reduce and thus detoxify NO_2 (Thauer et al., 1977; Moreno-Vivián et al., 1999; Latham et al., 2016).

1.2.1.3. Denitrification pathway in the rumen

Denitrification is another process for dissimilatory NO_3 reduction and consists of the conversion of NO_2 to NO, N_2O and N_2 (Figure 1.3) and despite evidence for the presence of the denitrifying genes (*nir, nor and nos*) in the rumen, this process has not been considered to appreciably contribute to ruminal NO_2 reduction (Jones, 1972; Kaspar & Tiedje, 1981; Leng, 2008; Latham et al., 2016). However, evidence of occurrence of denitrification in the rumen has been reported by de Raphélis-Soissan et al. (2017) with ^{15}N label from $^{15}NO_3$ appearing in N_2 in rumen gas.

The reduction of NO_2 to NO is catalysed by a copper-containing NO_2-reductase and a NO_2-reductase situated in the periplasmic space (Lundberg & Govoni, 2004). Both enzymes are present in the rumen microbiome (Brulc et al., 2009) and are inhibited by higher NO concentration (Averill, 1996). The nitrous oxide reductase enzyme reduces N_2O to N_2 and has been found in the bacterium *Wollinella succinogenes* which is present in the rumen (Simon, Einsle, Kroneck & Zumft, 2004).

1.2.2. Nitrate and nitrite absorption from the rumen and the lower gut

1.2.2.1. Kinetics of nitrate and nitrite absorption

Nitrate and NO_2 absorption across the rumen wall was apparent in the study of Pfander, Garner, Ellis and Muhrer (1957) on anaesthetized sheep dosed with NO_3 or NO_2 into the isolated rumen. A similar study was conducted by (Godwin et al, unpubl. data) and found that, after a solution of potassium nitrite was administered intraruminally, NO_3 and NO_2 appeared in blood suggesting that uptake of NO_2 from the isolated rumen did occur, although quite slowly (Nolan et al., 2016). Stumpff (2011) has shown that NO_3 was absorbed through the rumen epithelium of sheep using the Ussing chamber technique (Clarke, 2009). The idea of direct absorption of NO_3 and NO_2 through the rumen wall also arises from the rapid appearance of MetHb caused by the presence of NO_2 in the bloodstream after intraruminally administration of NO_3 (Lewis, 1951a; Wang et al., 1961). Furthermore, Würmli, Wolffram and Scharrer (1987b) suggested that NO_3 is probably absorbed across the rumen epithelium by the chloride and bicarbonate exchange mechanism.

The absorption sites of NO_3 and NO_2 from the gut have been more extensively studied in non-ruminants than in ruminants (Würmli, Wolffram & Scharrer, 1987a). In particular, Friedman, Greene and Epstein (1972) reported that sodium NO_2 was rapidly absorbed from the stomach of mice after oral administration even when a ligation of the gastroduodenal junction was performed. Grudziński (1991) found that only 10% of NO_2 was absorbed in the perfused intestine of the rat. In humans, Hunault, van Velzen, Sips, Schothorst & Meulenbelt (2009) found that 95–98% of an orally administered dose of NO_2 was absorbed in the intestine. The possibility of NO_3 and NO_2 being absorbed in the gastrointestinal tract of ruminants, as ocurrs in humans, needs further investigation.

1.2.3. Nitrate and nitrite appearance in the bloodstream

1.2.3.1. Plasma nitrate metabolites

The rate of absorption of NO_3 and NO_2 is low in non-ruminants (pigs), since minor amounts (10-20%) pass from the stomach and the rumen respectively to the bloodstream as NO_2 relative to rodents and humans with higher oral rates of absorption (90-95%) of both NO_3 and NO_2 (EFSA, 2009; Cockburn et al., 2013). Schneider and Yeary (1975) reported volumes of

distribution for plasma NO_2 of 192 mL/kg BW in the pony, 278 mL/kg in the sheep and 1624 mL/kg BW in the pony after intravenous administration of 20 mg/kg BW of $NaNO_2$. Contrary to NO_2, values reported for the volume of distribution for blood plasma NO_3 were lower and ranged between 210 and 330 mL/kg BW in humans, dogs, sheep, ponies, and goats (Schneider & Yeary, 1975; Schultz, Deen, Karel, Wagner & Tannenbaum, 1985; Lewicki, Wiechetek, Souffrant, Karlik & Garwacki, 1998; EFSA, 2009).

In plasma, NO_2 remains stable for several hours while in whole blood it is rapidly oxidised to NO_3, with a half-life in blood from 110 s to 20 min in humans (Wagner, Schultz, Deen, Young & Tannenbaum, 1983; Bryan, 2006) and 12 min in sheep (Schneider & Yeary, 1975). Contrastingly, NO_3 has a circulating half-life of 5-8 h (Wagner et al., 1983; Bryan, 2006). This difference in stability may explain in part why concentrations of NO_3 reported in plasma of ruminants were usually greater than plasma NO_2 for diets supplemented with NO_3 in ruminants (El-Zaiat et al., 2014; de Raphélis-Soissan et al., 2017; Lee, Araujo, Koenig & Beauchemin, 2017a).

1.2.3.2. Blood methaemoglobin

The ingestion of diets supplemented with NO_3 by ruminant livestock may cause accumulation of ruminally produced NO_2 in the blood which can reduce haemoglobin (Hb) to methaemoglobin (MetHb) and decrease the ability of the red blood cells to transport oxygen. A threshold for safe dietary NO_3 inclusion cannot be accurately established because of the large variation between animals (Cockrum, Austin, Kim, et al., 2010). In ruminants, a small portion of ruminal NO_2 (~10%) is absorbed into the bloodstream and distributed in the plasma, binding to erythrocytes. Nitrite is then converted to NO or NO_3 resulting in the formation of MetHb (Bradley, Eppson & Beath, 1939; Bruning-Fann & Kaneene, 1993; Cockburn et al., 2013; Vega-Villa, Pluta, Lonser & Woo, 2013; Lund et al., 2014). The normal physiological concentration of MetHb in ruminants is less than 1% of total Hb (Power et al., 2007) and levels between 20-50% may cause methaemoglobinaemia (Bruning-Fann & Kaneene, 1993).

1.2.3.3. Individual variability

In the study of Bruning-Fann and Kaneene (1993) a large inter-animal variation was reported in the amount of MetHb induced in animals when given the same dose of NO_3. The largest influencer of this variability is the rumen endogenous microbial population and their enzymatic capacity (Latham et al., 2016). Even though the rumen microbiota can adapt to dietary NO_3

(Lee & Beauchemin, 2014) with a subsequent increase in NO_3 and NO_2 reductase activity (Alaboudi & Jones, 1985; Miao Lin et al., 2013; Asanuma et al., 2015; Wang et al., 2018; Welty et al., 2019); NO_2 reduction to NH_3 appears to be more difficult to adapt and thus, increases the propensity to NO_2 accumulation (Lin, Schaefer, Zhao & Meng, 2013). Individuals can be identified as being lowly or highly tolerant to increased dietary NO_3 related to their performances, toxicity signs and differences in hepatic gene expression (Cockrum, Austin, Ludden & Cammack, 2010).

1.2.4. The contribution of rumen protozoa to nitrate reductase activity in the rumen

1.2.4.1. The role of rumen protozoa in nitrate reduction

In the studies of Yoshida, Nakamura and Nakamura (1982) and Nakamura and Yoshida (1991) it was reported that rumen protozoa accelerate NO_3 reduction when co-cultured with bacteria, and it was also suggested that rumen protozoa had greater ability to reduce NO_3 and NO_2 than the bacterial fraction. In contrast, Alaboudi and Jones (1985) stated that the activity associated with the protozoal fraction of the rumen microbiota may be due to the stimulation of bacterial activity provided by the protozoa such as by metabolic hydrogen, since hydrogen is a very effective reductant for NO_3 *in vitro* (Lewis, 1951b; Jones, 1972). Allison and Reddy (1984) did not find NO_3 or NO_2 reducing activity in the protozoal fraction. However, subsequently and in agreement with the results obtained by Yoshida et al. (1982) the ability of the rumen protozoa fraction to reduce NO_3 without accumulating NO_2, has been demonstrated *in vitro* when compared to whole rumen fluid or bacteria fraction, even when they were not adapted to dietary NO_3 (Lin, Schaefer, Guo, Ren & Meng, 2011). More recently Nguyen, Barnett and Hegarty (2016) reported that protozoa-free lambs consuming a 2% NO_3 diet had higher MetHb levels than did faunated lambs, indicating a possibly slower reduction of NO_3 to NH_3 compared to faunated lambs and suggesting a greater susceptibility to NO_2 toxicity in defaunated sheep. It appears a symbiotic relationship may exist between protozoa and associated bacteria, whereby both reduce NO_3 but protozoa mainly reduce NO_2 (Lin et al., 2011; Yang et al., 2016). However, protozoal metabolism may be self-limiting during high rates of NO_3 ingestion because the accumulation of NO_3 or its reduction products in rumen contents seem to inhibit protozoal growth (Sar et al., 2005; Nolan et al., 2016).

As for the effect of protozoa on NO_3 metabolism, the effect of NO_3 on ruminal protozoa is inconsistent in the literature. Occasionally, NO_3 has been reported to reduce the number of protozoa in the rumen when fed alone (Sar et al., 2005; Asanuma et al., 2015) or in combination with saponin plus sulfate or lineseed oil (Morgavi, Forano, Martin & Newbold, 2010; Patra & Yu, 2014) but this effect was not always observed (Nolan et al., 2010; van Zijderveld et al., 2010; Li, Davis, Nolan & Hegarty, 2012; Guyader, Doreau, et al., 2016; Nguyen et al., 2016; Popova et al., 2017). More research is needed to understand protozoal metabolism of NO_3 and NO_2 since they seem to play an important if unclear role in the safe of NO_3 supplementation and NO_3 and NO_2 effect on protozoa is also unclear.

1.2.5. Nitrate recycling pathway in the gastrointestinal tract

1.2.5.1. Circulating nitrate transferred into saliva

The oral cavity in humans contains a large number of NO_3-reducing bacteria that are equipped with highly efficient NO_3 reductases which use NO_3 for respiration or as a substrate for incorporation of N into biomass (Lundberg & Govoni, 2004). About 25% of all circulating NO_3 is actively taken up by the salivary glands and the commensal lingual flora generates NO_2 from NO_3 within saliva (Spiegelhalder, Eisenbrand & Preussmann, 1976; Lundberg, Weitzberg & Gladwin, 2008). In ruminants, the role of the salivary glands and lingual microbes in producing NO_2 is unknown but ruminating may also play a role by returning feed particles and rumen fluid containing NO_3 to the mouth where NO_2 could also be formed from NO_3 by microbial action of lingual bacteria (Leng, 2008). Evidence of recycling of blood NO_3 through the gastrointestinal tract (GIT) was reported in the isotope study of Lewicki et al. (1998) since ^{15}N appeared in urinary urea-N within 15 min and continued to increase post injection of ^{15}N-NO_3, indicating that NH_3 absorbed after enteric reduction of NO_3, was converted to urea in the liver and excreted in urine or recycled in the gut.

Despite the capacity of the bacteria in the distal GIT to reduce NO_2 in humans, (Tiso & Schechter, 2015), it is unlikely they contribute significantly to the enterosalivary circulation given that the majority of NO_3 and NO_2 are absorbed in the proximal small intestine in humans (Sobko et al., 2005; Koch et al., 2017). In the blood, NO_3 mixes with the NO_3 formed from the oxidation of endogenous NO produced from the nitric oxide synthases (*NOS*) enzymes. The NO_2 levels in plasma also increase after NO_3 ingestion (Lundberg & Govoni, 2004) because the enterosalivary circulation of NO_3 also leads to a sustained increase in plasma levels of NO_2,

and NO_3 may therefore be considered as a 'prodrug' of NO_2 with a slow-release profile (Lundberg et al., 2009).

1.2.6. Nitrate and nitrite uptake by tissues

Evidence of some storage of NO_3 within the body was supported by isotope dilution studies of Wang et al. (1961) in cattle and Lewicki et al. (1998) in sheep, who both observed a persistence of $^{15}NO_3$-N in plasma 8-50 h after isotope injection. When NO_3, NO_2 and NH_3 enter the bloodstream, they may be metabolized to other compounds, eliminated via the kidneys or recycled via saliva and other secretions to the gut and utilized by gut microbes (Nolan et al., 2016).

In a study with pigs to elucidate the short term fate of inorganic NO_3 and NO_2 across a variety of organs after acute NO_3 administration, Eriksson, Yang, Carlström and Weitzberg (2018) found that the highest tissue levels of NO_3 and NO_2 were in the gut. In the same study, there was a significant presence of NO_2 in the liver and kidneys, and NO_3 accumulated in the small intestinal lumen and locally converted to NO_2, since gut bacteria have the capacity to reduce NO_3 in humans (Koch et al., 2017).

In the literature, the presence of NO_3 and NO_2 in organs, meat or dairy products differs between experiments. The lack of accumulation of NO_3 and NO_2 in rumen tissue, tongue and liver after 2 months of 1% NO_3 supplementation in fattening cattle has been reported by Doreau, Arbre, Popova, Rochette and Martin (2017), supporting the results obtained in dairy products by Guyader, Doreau, et al. (2016) with 1.8% NO_3 diets. In meat products, feeding 3% NO_3 to cattle increased the NO_3 content by 21 µg/g, but this level did not appear to be a hazard to human health (Davison, Hansel, Krook, McEntee & Wright, 1964). Nitrate or NO_2 residues were not detected in lamb meat after supplementing sheep with 27 g/kg DM encapsulated NO_3 (El-Zaiat et al., 2013). Hegarty et al. (2016) found significant NO_2 concentrations (10 mg/kg) in raw meat but not in cooked meat of cattle fed diets containing 0.45% NPN as calcium NO_3 or urea. The same authors found no evidence of NO_3 being present in raw or cooked meat, with all samples having concentrations lower than the analytical detection limits.

1.2.7. Formation of nitrous oxide in nitrate supplemented ruminants

Nitrous oxide is a strong greenhouse gas (GHG) having a global warming potential 298 times greater than carbon dioxide (IPCC, 2014). Nitric oxide is released as a side product during NO_2 reduction (Vine & Cole, 2011). Nitric oxide is reduced to N_2O by a specialized nitric oxide reductase in the microbial cytoplasm (Spiro, 2007). It has been estimated that the formation of N_2O when supplementing ruminants diminishes the antimethanogenic effect of NO_3 by 12% in sheep fed 1 kg of a diet containing 2% NO_3 (de Raphélis-Soissan et al., 2014). In the same way, Petersen et al. (2015) reported a 15% reduction of the net mitigation benefit of CH_4 mitigation in cows supplemented with 2.1% NO_3 in DM as a result of N_2O emissions.

1.2.8. Excretion of nitrate-N in urine and faeces

Nitrate appears to be a normal constituent of human urine (Mitchell, Shonle & Grindley, 1916). The amounts of NO_3 excreted in urine greatly exceed that typically ingested mainly due to endogenous biosynthesis of NO_3 (Green et al., 1981; Khatri, Mills, Maskell, Odongerel & Webb, 2017). Levels of NO_3 in human plasma are between 20 and 40 µM under fasting conditions, implicating endogenous production (DeMartino, Kim-Shapiro, Patel & Gladwin, 2018). About 80% of NO_3 filtered by human kidneys is pumped back into the blood stream by active transport but excretion is maximal after five hours and complete within 18 hours in humans (Walker, 1996; Cockburn et al., 2013). In contrast, urinary NO_3 excretion in ruminants is markedly lower. Fifty hours after an intravenous injection of $^{15}NO_3$ in sheep, Lewicki et al. (1998) found 38% of ^{15}N dose had been recovered in urine, suggesting that some of the remaining $^{15}NO_3$ was metabolized in the GIT or stored in tissues. Urinary NO_3 excretion in steers supplemented with 58 g NO_3-N/d was 0.17 g/d and NO_2 was not detected in urine (Lee, Araujo, Koenig & Beauchemin, 2015b). Urinary NO_2 excretion is rapid and extensive with no accumulation in tissues. Short biological half-lives for blood plasma NO_2 of approximately 30 min in sheep, dog and pony have been reported (Schneider & Yeary, 1975).

Nitrate-N excreted in urine and faeces increases in proportion to the amount of NO_3 ingested (Lee et al., 2015b) and with the increasing amount of dietary protein (Setchell & Williams, 1962). An increase in N distribution towards faecal excreta is desirable as N in manure is more stable and less prone to microbial denitrification and nitrification than NH_3-N from urinary urea (Cabeza Luna, 2017). However, it would also be illogical to add extra N into

protein rich diets that would inevitably increase the excretion of N by the animal, potentially leading to release of N oxides and CH_4 from the excreta (Leng, 2008).

Little NO_3 excretion in faeces (0.35 g NO_3-N/d) has been reported in cattle supplemented with 58 g NO_3-N/d (Lee et al., 2015b), this NO_3 probably resulted from ruminal escape or recycling into the lower gut. Faecal NO_3 or NO_2 in humans is also negligible (Bednar & Kies, 1994).

1.3. Physiological effects of nitrate metabolites and responses in animal performance

1.3.1. Effect of dietary nitrate on animal productivity

1.3.1.1. Nitrate and nitrite effects on rumen microbes and fermentation

It has been suggested that dietary NO_3 not only reduces CH_4 emissions by acting as a H_2 sink but also exerts a toxic effect towards rumen microorganisms, particularly methanogens which are sensitive to low concentrations of NO_2 (Cheng, Phillippe & Majak, 1988; Iwamoto, Asanuma & Hino, 2002; Zhou, Yu & Meng, 2012; Asanuma et al., 2015; Guyader, Tavendale, et al., 2016). However, there are several rumen microorganisms that can utilize it for growth (Cheng et al., 1988; Leng, 2008). When rumen fluid from sheep not adapted to NO_3 was incubated, the presence of NO_2 caused a reduction in the cellulolytic and xylanolitic microbes and consequently reduced *in vitro* digestibility (Marais et al., 1988). However, Iwamoto et al. (2002) reported that the presence of NO_3 favoured the growth of NO_3-reducing *Selenomonas ruminantium, Veilonella parvulla* and *W. succinogenes* because they obtain energy from NO_3 or NO_2 reduction and they were also less sensitive to NO_2. In addition, NO_3 can also reduce the number of ruminal protozoa (Sar et al., 2005) which are H_2 producers. This reduction in protozoa numbers may also contribute to the reduction of enteric CH_4 (Callaghan et al., 2014).

Some bacteria can adapt to NO_3 or its reduction intermediates, while others probably not. Because methanogens do not become adapted, NO_3 may be used, either alone or in combination with other substances, to achieve persistent mitigation of CH_4 emissions from the rumen (Zhou et al., 2012).

1.3.1.2. Effects of nitrate on feed intake and digestibility

A summary of the effects of several doses of dietary NO_3 during different intervention periods on DMI, DMD and animal performances is shown in Table 1.2. It has been reported that more than 1% NO_3 in DM may affect animal performance by reducing feed intake and weight gain (Marais et al., 1988; Bruning-Fann & Kaneene, 1993). However, results on the effect of dietary NO_3 on animal performance are not consistent in the literature (Lee & Beauchemin, 2014). Lee, Araujo, Koenig and Beauchemin (2015a) observed a 15% reduction in DMI when beef cows were restrictively fed (75% of *ad libitum* intake) diets of 4.8% and 5.8% of encapsulated NO_3 (3.9 and 4.9% NO_3 in dietary DM, respectively). More recently, Klop, Hatew, Bannink and Dijkstra (2016) reported a reduction on DMI in dairy cows fed NO_3 under a restricted feeding regime. Nitrate tastes bitter and may lower palatability of diets with high inclusion rates (Farra & Satter, 1971) and causes lower feed intake of N-adequate diets as has been alluded in previous studies (Hulshof et al., 2012; de Raphélis-Soissan et al., 2014; Lee, 2014; Lee & Beauchemin, 2014; Lee et al., 2015a; Hegarty et al., 2016). Nevertheless, the inclusion of NO_3 in the diet has not shown any adverse effect on DMI in other studies (Nolan et al., 2010; van Zijderveld et al., 2010; Li et al., 2012; Pal, Patra, Sahoo & Mandal, 2014; Veneman et al., 2014; Olijhoek et al., 2016) or on carcass quality (Newbold et al., 2014; Duthie et al., 2016).

Nitrate has effects in the rumen that may affect appetite and eating behaviour (Velazco, Cottle & Hegarty, 2014; Lee et al., 2015a; Hegarty et al., 2016). As feed intake is one of the most important factors influencing animal productivity, the inconsistent results of feeding NO_3 on animal performance may be contributing to the low implementation of this strategy by farmers as a means of reducing enteric CH_4 emissions. Hegarty et al. (2016), also reported a significantly lower ADG and carcass weight in feedlot steers supplemented with calcium nitrate compared with urea-fed steers.

1.3.2. Nitrate effects on digesta kinetics

1.3.2.1. Relationship between mean retention time and methane production

The rate of passage is a measure of the time during which a portion of digesta is exposed to the processes of mixing, digestion and absorption in the GIT; it can be defined as the mean retention time (MRT) of digesta (Faichney, 1993). Low CH_4 yield (MY) has been consistently associated with shorter MRT in sheep and also in a range of species including humans (Hegarty,

2004; Nolan et al., 2010; Goopy et al., 2014; Nguyen et al., 2016; Bond et al.). Some variation in MY of sheep relies on differences in rumen size and rate of passage (Pinares-Patiño, Ulyatt, Lassey, Barry & Holmes, 2003; Goopy et al., 2014). Variation in DMI, rumen volume, proportions of contents in the digesta fractions and MRT, all of which influence fermentation rate, are the likely source of variation in MY among animals and may provide the genetic basis of difference in CH_4 emissions (Bond et al., 2017).

Evidence suggests that the lower CH_4 yield achieved in animals supplemented with NO_3 is in concordance with a shorter MRT of the particle and liquid phase. Nguyen et al. (2016) reported a greater DMI associated with a shorter MRT of both rumen solute and particulate in sheep fed a N-deficient diet when supplemented with NO_3. The authors reported that a shorter MRT allowed the animals to consume more feed due to a reduced rumen fill constraint, faster passage and a greater fermentation.

1.3.2.2. Effects of nitrate and nitrite on vascular function and blood pressure

Dietary NO_3 is a precursor of NO_2 which is altered in the blood to NO to exert a vasodilatory effect in humans (Tsuchiya et al., 2005). The formation and absorption of NO_2 in the rumen after a meal rich in NO_3 brings about a drop in the blood pressure and the concomitant formation of MetHb; both responses lower the capability for oxygen distribution in the blood (Malestein et al., 1980). It has been reported that NO_2 at near physiological levels, as well as in a short term dietary supplementation reduced blood pressure in rats (Nyström et al., 2012). The vasodilating effects of low-dose NO_2 have also been reported in mice, sheep, dogs, primates and humans (Cosby et al., 2003; Hunter, Blood, White, Pearce & Power, 2003; Hunter et al., 2004; Dias-Junior, Gladwin & Tanus-Santos, 2006; Lundberg et al., 2008). More recently, Whatman, Godwin & Nolan, 2013 reported that the lowered blood pressure in anaesthetised sheep infused intravenously with NO_2 was independent of MetHb production. This results suggest that the reduction in oxygen carrying capacity in blood might be related with physiological effects of NO produced in the gut before the symptoms of absorbed NO_2 on MetHb formation are apparent in ruminants (Nolan et al., 2016). Future studies on determining NO production and absorption in the gut of ruminants fed diets supplemented with NO_3 are therefore recommended.

1.3.3. Effects of nitrate metabolites on blood glucose and insulin dynamics

1.3.3.1. The nitrate - nitrite - nitric oxide pathway

The inorganic anions NO_3 and NO_2 were previously thought to be undesired residues in the food chain with potentially carcinogenic effects, or as inert oxidative end-products of endogenous NO metabolism; however, in the past decade research has shown that NO_3 and NO_2 are physiologically recycled in blood and tissues in humans to form NO and other bioactive N oxides (Cosby et al., 2003; Lundberg et al., 2008).

There are two major sources of NO_3 and NO_2 in the body: the endogenous production of NO_3 and NO_2 via L-arginine-NO synthase pathway and the diet. Nitric oxide is endogenously produced from the amino acid L-arginine and molecular oxygen in a reaction catalysed by complex *NOS* (Moncada & Higgs, 1991; Weitzberg, Hezel & Lundberg, 2010). The reaction of NO with oxyhaemoglobin produces NO_3 and MetHb, whereas the oxidation of NO forms NO_2 in plasma (Figure 1.4; Lundberg et al., 2009). Both enzymatic and non-enzymatic pathways have been identified for NO production (Bahadoran, Ghasemi, Mirmiran, Azizi & Hadaegh, 2015). The bioactivation of NO_3 from dietary or endogenous sources requires initial reduction to NO_2, and because mammals lack specific and effective NO_3 reductase enzymes, this conversion is mainly carried out by commensal bacteria in the GIT and on body surfaces (Lundberg et al., 2008).

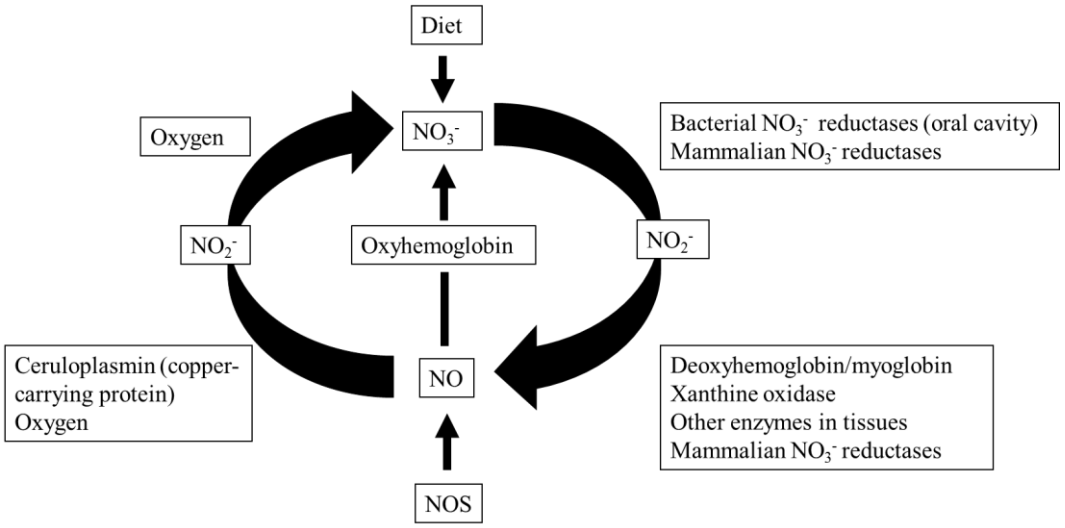


Figure 1.4. A mammalian nitrogen oxide cycle. Nitric oxide synthase (*NOS*) generates nitric oxide (NO) in cells to regulate a vast variety of physiological functions. The bioactivity of NO is acutely terminated by its rapid oxidation to nitrite (NO_2) and (NO_3). In the body NO_3 can undergo reduction to

NO_2 by bacteria in the oral cavity and by xanthine oxidase and possibly other enzymes in tissues. In blood and tissues NO_2 can be further metabolized to NO and other biologically active nitrogen oxides (not shown). This reduction is catalysed by various enzymatic and nonenzymatic pathways, most of which are greatly enhanced under hypoxic conditions. The reduction of NO_3 and NO_2 to NO completes a mammalian nitrogen oxide cycle. Adapted from Lundberg et al. (2009).

1.3.3.2. Nitric oxide, insulin secretion and regulation of blood glucose

Experiments conducted in humans and rats dosed with L-arginine stimulated insulin release and in parallel, it also elevated plasma glucose (Floyd, Fajans, Conn, Knopf & Rull, 1966; Jun & Wennmalm, 1994). Additionally, it was demonstrated that insulin release stimulated by L-arginine required the formation of nitric oxides (Schmidt, Warner, Ishii, Sheng & Murad, 1992). In the bioactivation of NO_3, NO_2 is an obligate intermediate (Lundberg & Govoni, 2004) and this more reactive compound is further metabolised to NO, via numerous enzymatic and nonenzymatic pathways in blood and tissues (Lundberg et al., 2008). Nitrate and NO_2 could increase insulin secretion by increasing pancreatic islet blood flow and activation of guanylyl cyclase and the cyclic guanosine monophosphate pathway; NO_3 and NO_2 also improve insulin resistance and glucose uptake by increasing gene expression of the insulin-regulated glucose transporter, GLUT4 (Nyström et al., 2012; Jiang et al., 2014; Bahadoran et al., 2015).

1.3.3.3. Early phase of glucose-stimulated insulin secretion

The abrupt rise in plasma glucose following intravenous glucose administration causes a rapid and transient increase in plasma insulin concentration (first-phase insulin secretion), which lasts for approximately 10 min and is followed by a slower, sustained rise in plasma insulin (second phase insulin secretion), which persists as long as plasma glucose remains elevated (DeFronzo, Tobin & Andres, 1979; Abdul-Ghani, Tripathy & DeFronzo, 2006).

It has been shown that the early phase of glucose-stimulated insulin secretion from pancreatic islets requires the formation of NO which may be produced in by a constitutively expressed *NOS* located in the vascular endothelium and islet alpha and delta cells, and subsequently diffuses to beta cells (Spinas et al., 1998; Spinas, 1999). Evidence that NO could be involved in insulin secretion was provided by Laychock, Modica and Cavanaugh (1991). The mechanism by which NO triggers insulin release is via increasing intracellular calcium through mobilization of calcium from intracellular pools such as the endoplasmic reticulum or from mitochondria (Spinas, 1999; Rahman et al., 2018)

1.3.3.4. Insulin signaling to nitric oxide

A positive relationship has been reported between basal vascular endothelial NO and insulin sensitivity, suggesting that under physiological circumstances, insulin causes arterial vasodilation in skeletal-muscle vascular beds (Petrie, Ueda, Webb, Elliott & Connell, 1996). By increasing its own delivery, and that of glucose to insulin-sensitive tissues, insulin may amplify its own action in promoting glucose uptake. Tessari et al. (2007) reported that insulin stimulated whole body oxidation and synthesis of NO products and decreased arginine turnover while increasing the fraction of arginine flux converted to NO oxidation products. Insulin binding to endothelial cells promotes NO production that can then activate the GLUT4 translocator in target cells independent of insulin (Jiang et al., 2014). Potential effects of NO on glucose uptake and insulin secretion have been suggested in humans, rodents and ruminants (Schmidt et al., 1992; Lundberg et al., 2009; Nyström et al., 2012; Turner, Godwin & Dobos, 2014). Insulin inhibits the degradation of glycogen and activates its synthesis from glucose in liver and in muscle tissue (Ruckebusch, Phaneuf & Dunlop, 1991) which is crucial in determining the final eating quality of meat after slaughtering (Tarrant, 1989).

1.3.3.5. Physiological effects of nitric oxide on animal production

Nitrite circulating in the blood is a precursor of NO that has profound physiological effects on tissue metabolism and animal production (Tsuchiya, Yoshizumi, Houchi & Mason, 2000; Nolan et al., 2016). Nitric oxide is also synthetized from L-arginine by *NOS* in all cells types (Wu & Morris, 1998). Dietary L-arginine supplementation was shown to increase muscle gain and reduced body fat mass via modulation of the arginine-NO pathway in growing-finishing pigs (Tan et al., 2009).

Because dietary NO_3 stimulates the synthesis of NO, ruminants fed a diet supplemented with NO_3 are highly exposed to the physiological effects of this molecule. Nitric oxide seems to be involved in the pathophysiology of the rumen as the exogenous stimulation of NO synthesis can potently diminish ruminal contractions (Onaga et al., 2001) and inhibits intestinal motility in sheep (Castro et al., 2012). Furthermore, Scala and Maruccio (2012) found evidence that NO cab obstruct rumination and reticular groove functioning. Similarly, Chen and Godwin (2013) reported a slower rumen digesta outflow rate caused by the simulation of *NOS*. For all these reasons, the reduction in feed intake frequently observed in NO_3-supplemented ruminants

may be related to the effects of NO in the GIT, although more research on this topic needs to be undertaken.

Most of the studies that investigated the effect of NO_3 on glucose and insulin dynamics have been focused on diabetes and obesity in humans. However, since insulin regulates blood glucose levels and nutrient uptake by muscle and adipose tissue, NO_3 addition in the diet of ruminants may have productive implications in meat quality and livestock productivity. The use of NO to improve meat quality is not new as reduction of NO_3 and NO_2 to yield NO has traditionally been used for curing meat, increasing shelf life and improve meat colour (Warner, Dunshea, Ponnampalam & Cottrell, 2005). Furthermore, potential effects of NO on meat quality of beef, lamb, pork and chicken muscles have been reported (Cook, Scott & Devine, 1998; Li et al., 2014; Cottrell, Ponnampalam, Dunshea & Warner, 2015; Liu et al., 2015). However, few studies have been conducted to investigate underlying mechanism of how NO affects meat quality and which pathways are involved (Liu, Warner, Zhou & Zhang, 2018).

1.3.3.6. Further aspects of nitric oxide effects on animal welfare

Evidence of NO synthesis by human skin cells was first reported by Heck, Laskin, Gardner and Laskin (1992). Nitric oxide plays a key role in orchestrating the skin's response to external stimuli such as heat, ultraviolet light, response to infection and wound healing (Cals-Grierson & Ormerod, 2004). Because NO is a potent vasodilator it has been proposed to play a role in pathophysiology of heat stroke (Hall, Buettner, Matthes & Gisolfi, 1994). The constitutive release of NO by the endothelial cells of the microvasculature plays an important role in setting the resting blood flow rate (Cals-Grierson & Ormerod, 2004). The vasodilating effect of NO is enhanced by local changes in temperature (Goldsmith et al., 1996) suggesting that *NOS* responds locally and rapidly to those changes and neurogenic signals, with an increase of NO production that has a direct effect on the vasculature (Cals-Grierson & Ormerod, 2004).

A protective effect after heat stress caused by the release of NO within the splanchnic circulation was observed in rats (Hall et al., 1994). Vasodilation is the physiological reaction of animals under heat-stressed conditions to dissipate increased heat via the skin (Sherwood, Klandorf & Yancey, 2012). However, supplementing cattle with 1% NO_3 in DM did not cause a significant amelioration of heat stress in beef cattle (Simanungkalit, 2015) and it is the only study performed in ruminants. The potential effect of NO_3 supplementation as a means of reducing heat stress effects in livestock is an important issue for future research.

1.4. Practical implications of nitrate supplementation of ruminants

Acceptance of feeding NO_3 by producers as a means of gaining carbon credits from reduced CH_4 output by their livestock will depend not only on the value of credits, but on whether they can achieve the nutritional benefits of NPN supplementation using NO_3 at a cost similar to urea supplementation, and with a minimal risk of NO_2 poisoning or other adverse effects on animal production (Nolan et al., 2016). However, NO_2 poisoning in cattle is principally governed by the amount and rate at which NO_3 is consumed by the animal (Benu et al., 2016).

1.4.1. Risk of nitrite toxicity in ruminants

1.4.1.1. Nitrate reduction rate in the rumen and nitrite toxicity risk

The risk of NO_2 toxicity is one of the main constraints to NO_3 supplementation, because it can be unpredictable (Callaghan et al., 2014) and individual animals also differ in their tolerance to NO_3 supplementation (Cockrum, Austin, Kim, et al., 2010). The different response to the NO_3 dose could be explained by inherent differences in microbial eco-systems or by factors associated with differential tissue metabolism, or both; and among-animal variation in the production of NO from NO_2 particularly could be involved (Nolan et al., 2016).

Blood MetHb increases when NO_3 concentration in the diet increases (Benu et al., 2016) but it has been also reported that blood MetHb levels increase over time (Godwin et al., 2015). It has been suggested that animals can adapt to low levels of oxygen in response to high MetHb levels (Jainudeen, Hansel & Davison, 1964). In addition, Godwin et al. (2015) reported that cattle increased the MetHb reduction rate in the erythrocytes after a chronic exposure to dietary NO_3^-. Therefore, understanding individual animal responses to NO_3, including both animal and microbial metabolic components is necessary to improve safety of NO_3 supplementation (Yang et al., 2016).

1.4.1.2. Adaptation of rumen microorganisms to nitrate diet

Several factors may influence the risk of NO_2 toxicity in ruminants, including animal species and age (Cockburn et al., 2013), dose rate of NO_3 intake and feeding frequency (Benu et al., 2016), the form of NO_3 administration (de Raphélis-Soissan et al., 2017), the type and presentation of the feed (Lee et al., 2015a), the diet composition (Bruning-Fann & Kaneene,

1993; Troy et al., 2015; Duthie et al., 2016) and the length of time NO_3 has been in the diet. Adaptation of animals to NO_3 is crucial since ruminants can adapt to NO_3 ingestion, provided the dietary introduction of NO_3 is sufficiently gradual to allow for microbial community adjustments (Farra & Satter, 1971; Miao Lin et al., 2013; Lee & Beauchemin, 2014). Furthermore, the rates of reduction on NO_3 and NO_2 increase by 3-10 fold when animals have been adapted to increasing levels of NO_3 in the diet (Allison & Reddy, 1984; Alaboudi & Jones, 1985). A stepwise inclusion of NO_3 in the diets of ruminants has shown changes in microbial populations in the rumen both *in vivo* and *in vitro* (Zhou et al., 2012; Miao Lin et al., 2013; Veneman et al., 2015; Capelari, Johnson, Latack, Roth & Powers, 2018).

Morgavi et al. (2010) suggested that rumen function will be disrupted if rumen CH_4 production is inhibited without the provision of an alternative hydrogen sinks. Sulphate-reducing bacteria, *Denitribacterium detoxificans* and *Wolinella succinogenes* use sulphate, nitrocompounds and nitrate, respectively, as electron acceptors (Weimer, 1998; Anderson & Rasmussen, 2000; Simon, 2002; Morgavi et al., 2010). *Wolinella succinogenes* grows by respiratory nitrate ammonification (Simon, 2002) and has the ability to reduce NO_3 into NH_3 with little accumulation of nitrites. *Wolinella* has also been shown to reduce CH_4 production *in vitro* but only if NO_3 was added to the incubation media (Iwamoto et al., 2002). These effects were also demonstrated *in vivo* using strains of Escherichia coli with high NO_3/NO_2-reducing activity (Sar et al., 2005a and 2005b).

It has been reported that cellulolytic ruminococci were sensitive to quite low concentrations of NO_2 Marais et al. (1988) and that NO_2 suppressed fermentation in general Iwamoto et al. (1999). However, if adaptation to dietary NO_3 was conducted carefully, no detrimental effects on feed intake or weight gain would occur (Lee and Beauchemin, 2014). In addition, Veneman et al (2015) evaluated the effect of NO_3 supplementation on rumen microbial community in dairy cows adapted to dietary NO_3 and concluded that rumen CH_4 emissions can be achieved without drastic effects on either rumen microbial population or its function.

1.4.1.3. Slow release forms of dietary nitrate

Encapsulated forms of NO_3 (EN) have been developed to release NO_3 slowly in the rumen to reduce the risk of NO_2 toxicity. Research shown that short-term effects of EN are favourable. Lee et al. (2015b) reported that EN reduced CH_4 production, increased dietary N utilization, decreased urinary N excretion compared to urea and slightly increased urinary and faecal NO_3

excretion, which decreased CH_4 production from manure. Similarly, de Raphélis-Soissan et al. (2017) conducted a short-term experiment with paraffin-wax-coated NO_3 salts that improved the safety of NO_3 supplementation in terms of lower blood MetHb concentrations. Long-term studies with EN have shown changes in eating behaviour without adverse effects on DMI, animal health and/or growth performance, and a tendency to reduce enteric CH_4 in the long term (Lee, Araujo, Koenig & Beauchemin, 2017b; Lee et al., 2017a).

1.4.2. Synergy of dietary nitrate and other methane abatement strategies

1.4.2.1. Lipids addition to the diet of ruminants

Lipids (fats and oils) are generally used to increase the energy density and reduce the dustiness of the diets of ruminants. The addition of fat can result in a persistent decrease in CH_4 emissions, without lowering animal production (Grainger & Beauchemin, 2011). The inhibitory response of fats depends upon concentration, type, acid composition and nutrient composition of diets (Machmüller, 2006; Beauchemin, Kreuzer, O'Mara & McAllister, 2008). Long chain fatty-acids such as sunflower oil and canola oil have been shown to reduce CH_4 emissions from cattle by up to 22% of gross energy intake (Beauchemin & McGinn, 2006). Generally, it is recommended that total fat should not exceed 6-7% of the dietary DM otherwise a depression in DMI may occur, negating the advantage of increasing the energy of the diet (Beauchemin et al., 2008; Patra, 2013).

Lipids reduce CH_4 emission by decreasing ruminal organic matter fermentation and supressing, the activity of methanogens and protozoal population. Lipids rich in unsaturated fatty acids also reduce H_2 availability by its use in hydrogenation of the fatty-acids (Johnson & Johnson, 1995). Polyunsaturated fatty acids, C12:0, C18:3 have more pronounced CH_4 supressing effects in cattle, while saturated long chain fatty acids are less effective (Patra, Lalhriatpuii & Debnath, 2016).

1.4.2.2. Enhancing the potential of nitrate to reduce methanogenesis through the combination of feed additives

One of the major constraints to NO_3 supplementation in ruminants is the risk of toxic effects due to NO_2 absorption. However, feeding NO_3 in combination with lipids, as well as including an adaptation period (Allison & Reddy, 1984) to the diet, appears to be a practical strategy that

should enable the livestock sector to safely mitigate enteric CH_4 emissions. In particular, using this combination strategy could be a way of reducing the amounts of NO_3 needed in the diet of ruminants, avoiding adverse effects on animals and obtaining higher levels of CH_4 reduction than those achievable from dietary NO_3 or lipids alone.

The combination of lipids and NO_3 emerges as a persistent and viable dietary option for mitigating CH_4 emissions as it has been reported effective both *in vivo* (Table 1.3) and *in vitro*. Linseed oil combined with NO_3 has been shown to reduce CH_4 emissions between 9% and 32% (Guyader et al., 2015; Doreau et al., 2017). More recently, differing effects of feeding NO_3 in combination with linseed oil on the composition and structure of ruminal and cecal microbiota have been reported (Popova et al., 2017; Popova et al., 2018).

When NO_3 and lipids were evaluated *in vitro*, results showed an additive effect on reducing methanogenesis (Patra & Yu, 2014; Patra & Yu, 2015). The mechanism of action of NO_3 and lipids in the rumen is different but both affect hydrogen availability for CH_4 formation in the rumen. Hydrogen may be oxidised during biohydrogenation of poly-unsaturated fatty acids (Johnson & Johnson, 1995) such as canola or linseed oil, while NO_3 directly inhibits the number of methanogens present (van Zijderveld et al., 2010) and acts as a hydrogen sink.

Table 1.3. Summary of effects of supplementing dietary nitrate (NO_3) in combination with linseed oil (LO), rapeseed oil (RO) or maize distillers dark grains (MDDG) on methane yield (MY, gCH_4/kg DMI), dry matter intake (DMI), dry matter digestibility (DMD) and animal performance: body weight gain (BWG; kg/d) or milk yield (L/d)

NO_3+ lipid (g/kg DM)	days	CH_4 yield	DMI	DMD	Performance	Reference
10 + 19 (LO)	60	ns	ns	na	BWG: -20%	Doreau et al. (2017)
21 + 24-37 (MDDG)	56	ns	ns	na	BWG: ns	Duthie et al. (2018)
18 + 35 (LO)	120	-30%	-17 % (P=0.07)	ns	Milk: -14% (P=0.08)	Guyader et al. (2016)
22 + 40 (LO)	70	-32%	ns	ns	na	Guyader et al. (2015)
21.5 + 27-53 (RO)	90	-7.5%	ns	na	na	Troy et al. (2015)
18 + 40 (LO)	na	-13-16%	na	na	na	Veneman (2014)

1.4.2.3. Cysteamine hydrochloride: a dietary additive to improve animal productivity with potential to reduce enteric methane emissions

Cysteamine is a biological compound that is produced in the GIT and hypothalamus of all animals that acts on the somatotrophic axis, regulating the concentration of somatostatin in the GIT, hypothalamus and circulatory system (Barnett & Hegarty, 2016). Somatostatin has inhibitory effects on growth, development, metabolism, nutrient absorption and digesta rate of flow through the GIT in ruminants (Barry, Faichney & Redekopp, 1985; McLeod, Harmon, Schillo & Mitchell, 1995). Through modulating plasma somatostatin, cysteamine has promoted growth and improved efficiency in a range of livestock species, such as broilers (Yang, Chen, Hong, Liu & Liu, 2006), pigs (Liu, Wang, Wu, Zhou & Liu, 2009), cattle (Wang et al., 2015) and sheep (Barnett & Hegarty, 2014, 2016).

Cysteamine has mainly been used as a dietary additive to improve growth and efficiency in livestock from intensive production systems. Recently, a reduction in enteric CH_4 has also been reported through the administration of CSH in sheep and cattle (Barnett & Hegarty, 2014; Huang et al., 2014; Sun et al., 2017). The mechanisms involved in CH_4 reduction through the addition of CSH in the diet of cattle may be related with a direct action of cysteamine on rumen protozoa and a reduction in propionate production observed *in vitro* (Sun et al., 2017). The authors hypothesized that the reason for the reduction in the number of protozoa was through the production of certain fatty acids, in response to CSH that are toxic to protozoa (Gao, Jing, Wang, Shi & Liu, 2016). Furthermore, CSH has been shown to affect the

smooth muscle and accelerate the rate of digesta passage in sheep (Barnett et al., 2012) which is inversely correlated with CH_4 production (Pinares-Patiño et al., 2011; Goopy et al., 2014). The effectiveness of CSH as a feed additive to improve the performance of ruminants while reducing CH_4 emissions requires more research.

1.5. Conclusions and research plan

Livestock CH_4 emissions are highly influenced by the demand for livestock products and at a global level, CH_4 emissions are forecasted to rise (Reay et al., 2018). Consequently, successful implementation of mitigation strategies is needed. Nitrate is an effective additive to reduce enteric CH_4 production in ruminant livestock while providing NPN for microbial growth. However, excessive quantities of NO_3 in the diet can expose animals to NO_2 toxicity. Feeding NO_3 in combination with fats (lipids & oils) offers an opportunity to reduce the amount of NO_3 included in the diet and therefore increase the safety of NO_3 supplementation. Nevertheless, the addition of lipids in the diets of ruminants may reduce DM digestibility and little is known of how this combination strategy affects digesta kinetics and nutrient outflow from the rumen.

The replacement of urea with NO_3 as an NPN source has been widely confirmed. However, much of the current research has been performed in N-adequate diets and more experiments to investigate the effectiveness of NO_3 supplementation to reduce CH_4 emissions in ruminants fed protein-deficient diets need to be undertaken. If NO_3 is recycled into the GIT in like manner to urea, further investigation is required and this should include the absorption sites and kinetics of absorption of NO_3 and NO_2 in the GIT of ruminants that are not clearly stablished.

Little evidence in the literature indicates that removal of protozoa from the rumen can increase susceptibility of animals to NO_2 poisoning. It is not well understood, however, how the interaction between rumen protozoa and bacteria affects NO_3 reduction in the rumen and the occurrence of methaemoglobinaemia.

Research on non-ruminant animals has demonstrated that dietary NO_3 and circulating NO_2 are precursors of the synthesis of NO which is an important signalling molecule and has profound physiological effects in mammals. Circulating NO increases insulin sensitivity in humans but little is known about potential effects of dietary NO_3 on glucose uptake and insulin metabolism in ruminants and possible beneficial effects on animal productivity.

The first experimental chapter of this thesis (Chapter 2) evaluates the effect of supplementing NO_3 in combination with canola oil on enteric CH_4 production, digesta kinetics and microbial N outflow from the rumen of cattle. It is hypothesised that feeding NO_3 in combination with canola oil may not only lead to a change in energy availability, but also due to differences in microbial N supply that could affect animal performance.

The third chapter aimed to evaluate the potential of dietary NO_3 to reduce CH_4 production in a highly protein-deficient diet fed to sheep. In parallel, a novel antimethanogenic additive, cysteamine hydrochloride was included. It was hypothesised that the inclusion of NO_3 or cysteamine in a protein deficient chaff-based diet could improve the productivity of animals while reducing their enteric CH_4 emissions.

Chapter four reports the contribution of ciliate protozoa on NO_3 reduction rate *in vivo* and *in vitro* in the rumen fluid of sheep. It was hypothesised that removal of protozoa from the rumen of sheep would increase NO_2 concentrations in the rumen fluid and plasma due to a slower NO_2 reductase activity. A greater susceptibility of defaunated sheep to produce high MetHb levels and NO_2 poisoning was also hypothesised.

The fifth chapter provides evidence of the kinetics of absorption of NO_3 and NO_2 in portions of the GIT of sheep and quantifies the fate of ^{15}N-NO_3 and ^{15}N-NO_2 in organs and tissues using ^{15}N as a tracer. It was hypothesised that NO_3 and NO_2 are directly absorbed through the rumen and small intestine epithelium whereas, because of acidic conditions, no absorption of NO_2 would occur in the abomasum. Rapid metabolism of intravenous ^{15}N-NO_3 and ^{15}N-NO_2 and appearance of ^{15}N in tissues and organs was also hypothesised.

The last experimental chapter of this thesis (Chapter 6) is focused on investigating the hypothesis that dietary NO_3 affects the insulin sensitivity in mammals. It was hypothesised that circulating NO_2, resulting from a low dose of dietary NO_3, would increase glucose uptake by increasing insulin release and insulin sensitivity.

Finally, an integration of the main results and conclusions of the five experimental chapters is presented in chapter seven. This general discussion integrates new findings with the literature and the hypothese presented in this thesis.

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Statement of originality

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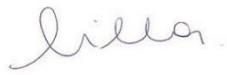
Name of Candidate: Maria Laura Villar

Name/title of Principal Supervisor: Professor Roger Hegarty



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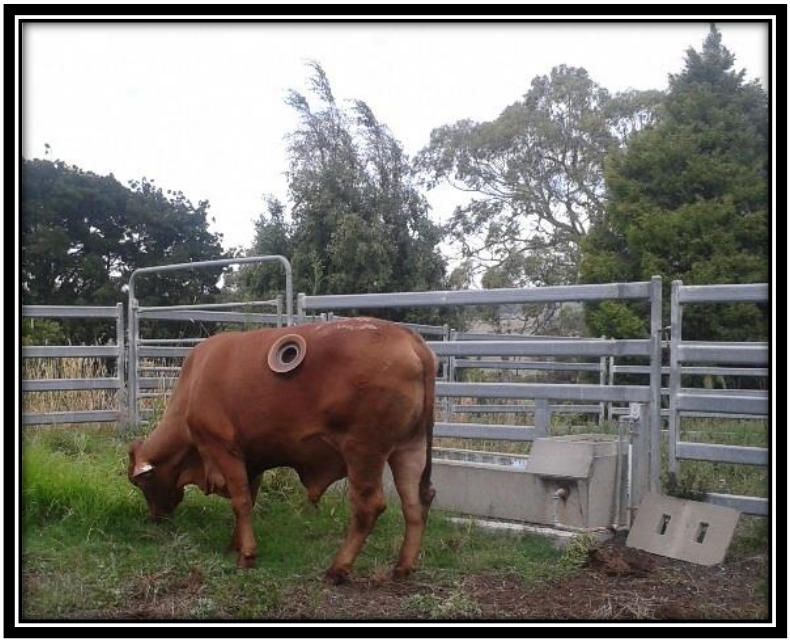
Chapter 2. The effect of dietary nitrate and canola oil alone or in combination on fermentation, digesta kinetics and methane emissions from cattle



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The effect of dietary nitrate and canola oil alone or in combination on fermentation, digesta kinetics and methane emissions from cattle

2.1. Abstract

The objective of this experiment was to evaluate the effects of nitrate and canola oil, alone or in combination, on enteric methane (CH_4), volatile fatty acid (VFA) concentrations and outflow of DM and microbial non-ammonia nitrogen (MicNAN) from the rumen of cattle. Nitrate and lipids have been recognized as effective dietary additives to reduce CH_4 production. Four rumen-cannulated steers were used in the experiment which was designed as 4 × 4 Latin Square with four 21-d periods and four treatments. Dietary treatments consisted of a control diet (CON: 400 g/kg lucerne chaff and 600 g/kg barley grain), NO_3 (CON + 20 g/kg nitrate), O (CON + 50 g/kg canola oil), and NO_3+O (CON + 20 g/kg nitrate + 50 g/kg canola oil) with all inclusions expressed as g/kg as-fed. Exogenous markers (Co-EDTA, Yb-acetate and $^{15}NH_4Cl$) were continuously infused into the rumen over 4-d to estimate digesta flow and rumen N outflow while whole tract digestibility (DMD) was determined using chromic oxide. Compared with the CON diet, feeding the NO_3+O diet reduced ($P<0.01$) methane yield (MY, g CH_4/kg DMI) by 25% and daily methane production (DMP, g CH_4/d) by 26% ($P<0.01$). Oil-containing diets reduced rumen volume ($P<0.01$) and the mean retention time (MRT) of particulate and liquid digesta ($P<0.01$). Total VFA did not differ between treatments ($P>0.05$) but nitrate-containing diets increased acetate proportion ($P<0.01$) whereas feeding the O diet increased propionate proportion ($P<0.01$). The rumen protozoa concentration was significantly reduced in cattle fed the O diet ($P<0.01$). This experiment demonstrates that the CH_4-mitigating effect of feeding NO_3+O in combination to cattle was more than the sum of their individual contributions and this was consistent with a reduction in rumen MRT; although an adverse effect on DMD was observed. Reducing methanogenesis by feeding NO_3+O in this experiment did not improve the flow of MicNAN from the rumen (g MicNAN/d), microbial growth efficiency (g MicNAN/digestible organic matter intake, DOMI) or the proportion of microbial N derived ruminal from rumen NH_3.

Key words: mean retention time, methanogenesis, rumen protozoa.

2.2. Introduction

Methane accounts for 43% of the greenhouse gas (GHG) emissions from livestock with the remaining emission released almost equally as nitrous oxide (29%) or carbon dioxide (27%) (Herrero et al., 2016). One strategy to reduce GHG emissions in ruminants is the manipulation of rumen fermentation by using dietary additives. Supplementing diets with nitrate reduces methanogenesis (Lee & Beauchemin, 2014) and strategies to reduce the risk of nitrite (NO_2) poisoning are being developed (de Raphélis-Soissan et al., 2017; Lee, Araujo, Koenig & Beauchemin, 2017a, 2017b). In addition to reducing the loss of energy in CH_4, nitrate can replace urea as a non-protein N source for rumen microorganisms (Leng, 2008; Goopy & Hegarty, 2018) and its inclusion in lick blocks to get carbon credits has been approved in Australia (DoE, 2013).

Lipid supplementation is another option to reduce enteric CH_4 emissions by supressing the growth of methanogens (Patra & Yu, 2012), or by lowering ruminal fermentability of the diet and to a lesser degree through hydrogenation of unsaturated fats (Beauchemin & McGinn, 2006). The inhibitory effect of fat supplementation on CH_4 emissions is not transitory but persists over time (Moate et al., 2011; Guyader et al., 2016). More recent studies have shown that the combination of dietary nitrate and dietary oils in cattle has an additive and longer-term CH_4 inhibiting effect than either treatment on its own (Guyader et al., 2015; Duthie et al., 2018).

It has been reported that a smaller rumen volume and faster ruminal digesta passage rate is associated with a reduction of CH_4 emission rate in sheep (Pinares-Patiño, Ebrahimi, et al., 2011; Goopy et al., 2014). However, there is no information about the effect of nitrate and oil in combination on digesta kinetics or MicNAN outflow in cattle. This experiment evaluated the effects of nitrate salts and canola oil, alone or in combination, on CH_4 production and VFA balance, outflow of microbial N from the rumen of cattle and microbial growth efficiency (g MicNAN/DOMI). We hypothesised that feeding NO_3+O diet may not only lead to a change in energy availability, but also due to differences in microbial N supply that could affect animal performance.

2.3. Materials and methods

All procedures involving animals were performed in accordance with the University of New England Animal Ethics Committee (AEC approval no. 15/104)

2.3.1. Animals, diets and experimental design

Four mature rumen-cannulated steers were used in the experiment, which was designed as a 4 x 4 Latin square with four 21-d periods and four dietary treatments in a 2 × 2 factorial arrangement. The complete 4 × 4 Latin square design conducted in this experiment provided 4 replicates per treatment and fully allows for between animal variations. Steers (698 ± 21 kg, mean ± SD) were randomly allocated to the dietary treatment and progressively adapted to the diet. Each steer was offered one of the four dietary treatments in each period. All diets were fed at 1% liveweight (LW) being 7.5 kg fresh feed/d to each animal. The control diet was a blended chaff mixture (40% lucerne chaff; 60% rolled barley grain) fed alone (Control; CON) or with inclusion of 20 g/kg nitrate (NO_3), provided as 31.4 g/kg calcium nitrate ($5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway). The third treatment (O) had 50 g/kg canola oil added to the chaff and the final treatment (NO_3+O) contained 20 g/kg nitrate and 50 g/kg canola oil in combination, with all inclusions expressed as g/kg as fed. Each experimental period was preceded by 4 d of adaptation to the experimental diet where 50% of the CON diet and 50% of the experimental diet was offered on Day 1 and Day 2; 25% of CON diet and 75% of the experimental diet was offered on Day 3 and Day 4, and from Day 5 to Day 21 cattle were fed the experimental diets (Table 2.1). While diets were not isonitrogenous, the level of crude protein (CP) relative to metabolise energy (ME) in the control diet (1.9 g N/MJ ME) was above that required to maximise rumen microbial activity (Freer, Dove & Nolan, 2007) so the additional N provided by nitrate was unlikely to stimulate microbial growth or fermentation. Diets were prepared in a ribbon mixer every two weeks by spraying dissolved Bolifor or canola oil onto the chaffed lucerne hay and barley grain so that these additives could not settle out when fed in each period. Animals were offered 7.5 kg/d of their experimental diet in two meals each day (at 0930 and 1700 h). During days when markers were infused the daily ration was offered in three equal portions at 0730, 1500 and 2300 h (Table 2.1). Over the 3 day of infusions, reticular and rumen samples were scheduled to provide samples matching each hour over the 8 hour interval between feeds, associated with feeding 3 times per day. This was done to minimize diurnal

fluctuation in fermentation and provide a very stable rumen fermentation and marker concentration in digesta samples.

Table 2.1. Experimental schedule for animal adaptation, feeding, marker introduction and sampling for each experimental period.

Day	Feeding (F)/Sampling (S)	Activity
1-4	(F) 0900, (F)1700 h	Gradual adaptation to the experimental diet.
5	(F) 0900, (F)1700 h	100% experimental diets were fed from day 5 up to day 21.
11	(F) 0900, (F)1700 h	Inclusion of chromic oxide in feed commenced. Animals fed twice daily.
14	(F) 0900, (F) 1700 h	Measurement of enteric CH4 emissions in respiration chambers.
15	(F) 0730 , (S) 1000, (S) 1300, (F) 1500, (F) 2300 h	Inclusion of chromic oxide in feed three times per day. Rumen digesta background samples and faeces collected. Live weight was recorded.
16	(F) 0730, (S) 1000, (F) 1500, (F) 2300 h	Intraruminal infusions commenced
17	(F) 0730, (F) 1500, (F) 2300 h	Intraruminal infusions continued
18	(F) 0730, (S)1000, (F) 1500, (S) 2200, (F) 2300 h	Reticular, rumen and faecal samples collected
19	(S) 0730, (F) 0800, (F) 1500, (S)1600, (S) 1900, (F) 2300 h	Reticular, rumen and faecal samples collected
20	(S) 0400, (F) 0730, (S) 0900 h	Reticular samples collected. Intraruminal infusion ceased
	(S) 1300, (S) 1500, (F) 1530, (S) 1900, (F) 1930 h	Rumen digesta and faecal samples collected (post-infusion). Chromic oxide supplementation cease
21	(F) 0900, (F) 1700 h	Measurement of enteric CH_4 emissions in respiration chambers. Live weight was recorded after 24 h.

Feed offerings and refusals were weighed and recorded daily throughout the experiment and mean daily intake was calculated. Samples of each diet were collected during each experimental period, stored at -20°C and analysed for chemical composition (Table 2.2) at the NSW DPI Feed Quality Service, Wagga Wagga Agriculture Institute. The methods of AFIA (2014) used specifically were Acid detergent fibre, neutral detergent fibre DMD of the feed and crude fat estimated by petroleum ether extraction. Metabolisable energy was calculated based on method 2.2R. Feed CP was determined by Dumas combustion (AOAC, 990.03). Energy values were determined using a bomb calorimeter

(Method ID LMOP 2-1118). Diets were formulated to meet the maintenance requirements of beef cattle (Freer et al., 2007). From Day 1 to Day 13 of each period animals were housed individually in pens (3 m x 2 m) equipped with a feeder and water bowl. Before the morning feeding on Day 14, animals were moved to four open circuit respiration chambers for measurement of CH_4 emissions over 24 h. On Day 15, animals were weighed, background rumen digesta samples were collected, cannula bungs were equipped with infusion lines and animals were constrained individually in smaller pens (2 m × 1.5 m) for the following 5 d. Digesta marker infusion and rumen-reticulum sampling were performed from Day 16 to Day 20. Before the morning feeding on Day 21, animals were again moved to respiration chambers for a second 24 h period of CH_4 emission measurement and the final LW of animals was recorded (Table 2.1).

2.3.2. Infusion of flow markers (Co-EDTA, Yb-acetate and $^{15}NH_4Cl$)

Animals received a continuous intraruminal infusion of ytterbium acetate (14.64 g/d of Yb, Inframat, 99.99%), ^{15}N-ammonium chloride (3.82 g/d of ^{15}N, 99.9 atoms %, ICON Isotopes, MI, USA) and Co-EDTA (16.70 g/d of Co, AVA Chemicals, Mumbai, India) for 4 d, commencing at 0900 h on Day 16 and ceasing at 0900 h on Day 20. Markers were dissolved in MilliQ® water (5.7 mg Co/L, 1.1 mg ^{15}N/L and 4.9 mg Yb/L) and continuously infused at rates of 0.19 ml/min (Co), 0.21 ml/min (Yb) and 0.20 ml/min (^{15}N) , using a peristaltic pump (Masterflex L/S 7528-30, Cole-Parmer, USA). Before beginning the infusions in each period, two samples of mixed rumen contents were taken from each steer and stored at -20°C for later determination of background ^{15}N, Co and Yb concentrations. Chromic oxide was well mixed in the total diet and fed daily from Day 10 to Day 21 in each experimental period to assess whole tract digestibility (Table 2.1).

Table 2.2. Nutrient composition of the experimental diets fed to cattle (g/kg DM).

	CON	NO_3	O	NO_3+O
Added nutrients				
Bolifor[1] (g/kg as-fed basis)	-	31.40	-	31.40
Nitrate provided as Bolifor[1] (g/kg as-fed basis)	-	19.97	-	19.97
Canola oil (g/kg as-fed basis)	-	-	50	50
Analysed nutrient composition				
DM	917	901	923	905
DM digestibility	778	773	775	768
Digestible organic matter	770	763	768	758
Organic Matter	950	940	950	940
Neutral detergent fibre	303	290	298	290
Acid detergent fibre	155	150	158	150
Crude protein	140	167	138	166
Crude fat	19	19	73	80
Metabolisable energy (MJ/kg DM)	12.0	11.9	13.4	13.5
Gross energy (MJ/kg DM)	18.6	18.1	19.6	19.2

[1]Bolifor: $5Ca(NO_3)_2.NH_4NO_3.10H_2O$ (636 g/kg nitrate in Bolifor)

2.3.3. Sampling for digesta flow, rumen bacteria, VFA concentrations, rumen ammonia (NH_3-N) and protozoal enumeration.

Reticular samples (7 per animal) were collected via the rumen cannula at 1000 and 2200 h on Day 18, at 0730, 1600 and 1900 h on Day 19, 0400, and 0900 h on Day 20 (Table 2.1). This was at least 14 d after animals had been adapted to their full diet which is considered sufficient to stabilize the rumen biota (Henderson et al., 2015). Reticular sampling was conducted as described in Hristov (2007). Samples were collected using a 30 cm × 65 mm PVC pipe capped at both ends with a 70 mm × 35 mm port cut into the side. The sampling pipe was fitted with a wider diameter sleeve that could be slipped up or down the collection tube to cover or expose the sampling port. The sampler was closed, held in the right hand and introduced through the rumen cannula and pushed forward into the reticulum until the proximal wall of the reticulum could be felt. The sleeve was then pulled up to expose the sample port and the sampler moved around within the reticulum, to allow digesta to enter the sampler. Then the sleeve was lowered to cover the port before the sampler was removed

from the rumen. The reticular digesta was then tipped into a beaker and mixed thoroughly before a 40 ml subsample was collected and stored at -20°C.

Rumen digesta samples (10 per animal were collected via the rumen cannula using the same technique as for reticular digesta sampling. Rumen background samples were collected at 1000 and 1300 h on Day 15. Samples were taken at 1000 and 2200 h on Day 18, at 0730, 1600 and 1900 h on Day 19 and at 0400, 0900 1300, 1500 and 1900 h on Day 20. The last 3 samples taken on Day 20 were to enable the post-infusion decline in marker concentrations to be monitored (Table 2.2). At every sampling time, a 100 ml of rumen digesta was collected. A 50 ml subsample of whole digesta was stored at -20°C. A second 50 ml subsample was filtered through a double layer of cheesecloth muslin into a beaker. Rumen pH was measured immediately using a portable pH meter (Ecoscan pH6, Eutech Instruments Singapore). A further 10 ml subsample of rumen fluid was taken and acidified with 0.3 ml of 18 M sulfuric acid and then frozen at -20°C for subsequent VFA and NH_3 analyses. A final 15 ml rumen fluid subsample was centrifuged at 18000*g* for 20 min in a refrigerated centrifuge (Velocity 30R, Dynamica Pty Ltd, Australia) and the supernatant liquid was discarded. The topmost (whitest) layer of bacteria on the particulate pellet was rinsed with 25 ml of MilliQ® water and transferred to another centrifuge tube. These bacteria were re-suspended in MilliQ® water and again centrifuged. This washing procedure was repeated 2 more times. The final pure bacteria pellet was taken up in 3-5 ml of MilliQ® water, frozen at -20°C and then freeze-dried for N and isotope analysis. Freeze-dried samples of rumen bacteria containing approximately 80 µg of N were weighed into 8 x 5 mm tin capsules prior to analysis (Sercon Limited, Gateway, Crewe, Cheshire, UK). Rumen $^{15}NH_3$-N from rumen fluid was recovered by diffusion from thawed rumen fluid samples in clean sealable glass bottles (McCartney bottle, Wide Mouth, 28 ml) as described by de Raphélis-Soissan et al. (2017) .

The abundance of ^{15}N in rumen bacteria and rumen NH_3-N was analyzed with a Sercon 20-22 continuous flow isotope-ratio mass spectrometer connected to an ANCA-GSL sample preparation unit (Sercon Limited, Gateway, Crewe, Cheshire, UK). Irreversible loss rate (ILR) of rumen NH_3 was calculated by dividing the average (plateau) enrichment of the 10 rumen NH_3 samples from Day 18 to Day 21 by the infusion rate of ^{15}N-NH_3 (mmoles ^{15}N/min).

Frozen samples of acidified rumen fluid were thawed at room temperature and centrifuged at 15000*g* for 10 min. A 0.5 ml subsample of supernatant was analysed for ruminal VFA concentrations by gas chromatography using isocaproic acid as the internal standard with a Varian CP-3800 chromatographer and Varian Star integration software as described by Nolan, Hegarty, Hegarty, Godwin and Woodgate (2010). A second 0.5 ml subsample of supernatant was diluted (1 in 20) in MilliQ® water and rumen NH_3-N was determined by a modified Berthelot reaction using a continuous flow analyser (SAN ++, Skalar, Breda, Netherlands).

At the time of collection, a 4 ml subsample of rumen fluid was placed in wide-neck McCartney bottle containing 16 ml of formaldehyde-saline (4 % formalin v/v) for protozoa enumeration. Protozoa were counted with a Fuchs-Rosenthal optical counting chamber (0.0625 mm^2 and 0.2 mm depth) using a staining technique based on the procedure of Dehority (1984). Protozoa were differentiated into Isotrichidae and Entodiniomorph families and by size into large (> 100 µm) and small (< 100 µm) protozoa.

2.3.4. Dry matter content, digestibility and digesta kinetics

Dry matter (DM) content of feed, rumen digesta, reticulum digesta and faecal samples were determined by drying at 65°C in a fan-forced oven to a constant weight and then ground through a 1 mm sieve.

Total tract DMD was estimated using DM intake (DMI) results and faecal concentration of the indigestible marker Cr_2O_3. Fifteen grams of chromic oxide (10.2 g Cr) was mixed onto the feed of individual animals. The marker was provided twice daily (7.5 g Cr_2O_3 × time at 07.30 and 17.00) from d 10 to d 14 during the adaptation period and then 3 times daily (5 g Cr_2O_3 × time at (0730, 1500 and 2300 h), from Day 15 to Day 21 during the infusion period. Although most diets were fully consumed, if any feed was refused, the daily Cr intake was assumed to drop by the same proportion. Faecal samples (approximately 55 g of wet weight) were collected twice daily (0900 and 1730 h) from the clean floor of each pen from Day 10 to Day 14 and three times a day (0730, 1500 and 2300 h) from Day 15 to Day 20. An additional faecal sample was collected prior to commencing Cr_2O_3 feeding in each period and was used to determine the background Cr concentration. Chromic oxide was assumed to be completely indigestible, and the digestibility of DM was calculated as follows:

DMD = 1- [Cr eaten (mg/d)/DMI (kg/d)]/Cr in faeces (mg/kg of DM)

Small amounts (< 10 g) of the ground feed, faecal and digesta samples containing the markers were analyzed for Cr concentration using a Bruker Tracer III-V portable X-ray Fluorescence (pXRF) with associated software (Bruker Corp., Billerica, Massachusetts, USA). The procedure for measuring markers concentrations using XRF calibration curves has been published by Barnett et al. (2016).

The kinetics of rumen digesta were determined from the concentrations of markers (Co and Yb) in the ten samples of rumen contents collected during the infusion period (Day 18 – Day 20 as previously chronicled) and in the three samples from after the infusions ceased (Day 21). Concentration versus time data were fitted using non-linear curve fitting algorithms of WinSAAM models (Stefanovski, Moate & Boston, 2003) with one compartment representing either rumen fluid or rumen particulate DM. With knowledge of the rates of infusion of Co (fluid marker) the parameters for the curves of best fit gave estimates of the rumen fluid volume (litres) and the outflow rates of fluid (litres/d).

2.3.5. Preparation of reticulum digesta samples and digesta flow calculations

A single bulked sample of reticular digesta was made for each animal in each period by mixing an equal weight of material from each of the 7 samples. Sodium tetraborate (1 g/16 ml sample) was added to increase sample pH and facilitate removal of NH_3. The mass of sodium tetraborate added was recorded and subtracted when determining net DM content. The bulked sample was fractionated into a mostly fluid phase (FP) and a mostly particulate phase (PP) by allowing the digesta to settle. A subsample of each phase was taken, freeze dried and ground through a 1 mm sieve for marker concentration analysis by inductively coupled plasma optical emission spectroscopy (ICP-OES; Varian Vista Radial MPX, Varian Medical Systems, Palo Alto, California, USA). Reticular digesta flow was calculated using the double marker method of Faichney (1992). Due to their high concentrations, reticular markers in Animal 4 in Period 3 for OIL diet and Animal 1 in Period 3 for NO_3+O diet were determined using a Bruker Tracer III-V portable XRF. All other calculations were done using marker concentrations estimated by ICP-OES. Marker concentration in both FP and PP, corrected for marker concentration in background samples, were used for calculation of reconstitution factors, i.e. the number of units of FP that must be added (or removed from)

one unit of PP to obtain true digesta (TD). Dry matter apparently digested in the rumen (DMADR) was calculated by deducting outflow of DM from DMI. The ^{15}N, enrichment ratio, enrichment in TD/enrichment in bacterial was used to determine the fraction of non-ammonia N (NAN) flow in total NAN leaving the rumen and then microbial NAN outflow (MicNAN). Microbial NAN outflow was calculated by multiplying TD flow and NAN content in TD (mg N/kg TD) by the enrichment ratio. Organic matter (OM) digested in the rumen (OMDR) was estimated assuming 950 g/kg OM in DMADR. Microbial NAN (MicNAN) being microbial NAN flow and digestible OM intake (DOMI) were used to estimate microbial growth efficiency as g MicNAN/kg DOMI (Broderick & Merchen, 1992).

2.3.6. Measurements of methane production

In each period, DMP of the cattle was estimated over 24 h when they were confined in open circuit respiration chambers with data averaged for the 2 measures for each animal in each period. Two 24 h measures separated by time were chosen as the correlation between consecutive days is very high but declines over longer period (Herd et al., 2014; Pinares-Patiño et al., 2013). During Period 1, DMP was only measured on Day 13. For Periods 2, 3 and 4 DMP was measured on Day 13 and Day 21. Chambers were opened and resealed daily at 0900 and 1700 h when cattle were fed. Recovery of CH_4 through the chambers was checked pre-measurement and post-measurement by introducing pure CH_4 at a known rate via a mass flow controller (Smart Trak 2 Series 100, Sierra Instruments, Monterey, CA, USA) and a Servomex analyser was used to quantify CH_4 concentration data. All daily CH_4 emission data was corrected for 100% CH_4 recovery (mean 108% ± 1.8%). Full details regarding the use of these open-circuit respiration chambers at Armidale (UNE) and CH_4 measurements protocols have been described by Hegarty, Bird and Woodgate (2012).

2.3.7. Statistical analysis

Variables with several data were averaged per animal and per Period and the effect of dietary treatments was determined using a linear mixed-model that included the random effect of animal (n = 4) and the fixed effects of Period (n = 4), Nitrate (CON and OIL versus NO_3+O), Oil (CON and NO_3 versus NO_3 and NO_3+O), and the interaction Nitrate x oil. All statistical tests were carried out in R 3.3.1 (R Core Team, 2016) using the package *lme4* for linear mixed models. During the course of the experiment, animal 4 (NO_3+O diet in Period 1) left

a large feed refusal while it was inside of the respiration chamber and animal 1 (NO_3 diet in Period 2) lost the cannula bung before it was scheduled to go into the chamber and lost a large amount of rumen fluid. Thus, observations of those animals for DMP and CH_4 yield (MY) were removed from the CH_4 dataset for those periods. Because of marker infusion problems observations from animal 3 in Period 1 for O diet and animal 4 in Period 1 for NO_3+O diet were removed from the analyses of digesta flow. Homogeneity of variance and normal distribution were tested using residuals plots and Shapiro-Wilk test. Main effects of NO_3, O and the interaction Nitrate x Oil were tested using contrasts and multiple comparisons (lsmeans procedure) were performed when the interaction Nitrate x Oil was tested significant. Differences with $P < 0.05$ were regarded as significant and $P < 0.10$ as a trend.

2.4. Results

2.4.1. Feed intake and dry matter digestibility

Feeding NO_3+O decreased DMI relative to the O treatment (P<0.01, Table 2.3). Compared with cattle fed the CON diet, feeding NO_3 alone or in combination with canola oil reduced DMD between 4 and 6 g/100g DM respectively (P<0.01).

2.4.2. Methane production and rumen fermentation

The CH_4-mitigating effect of Nitrate and oil (Nitrate x oil, P=0.12; Table 2.3) was more than additive than that of NO_3 or O diets fed separately. Feeding NO_3+O diet, reduced MY relative to cattle fed the other diets (P<0.01) and no differences were observed between CON, NO_3 and O treatments (P>0.05). There was a tendency towards a positive correlation between DMD and MY in the 3 experimental periods for NO_3+O treatment (DMD = 0.53 + 0.01 MY, r = 0.99, P=0.09).

Mean ruminal pH for the dietary treatments ranged from 5.98 to 6.30. The O treatment significantly reduced rumen pH compared to NO_3 or NO_3+O treatments (P<0.01). Nitrate and oil-containing diets increased rumen NH_3-N concentrations (P<0.01; P=0.06) relative to CON.

Concentrations of total VFA in the rumen did not differ between dietary treatments (P>0.05; Table 2.3). However, nitrate inclusion increased acetate proportion ($P < 0.01$) and acetate to propionate ratio (A:P; P<0.01); whereas these parameters were reduced by feeding

the O diet. Propionate proportion was greater in cattle fed the O diet compared with the other diets ($P<0.01$) whereas butyrate was greater in the O treatment relative to NO_3-fed cattle ($P<0.01$). Feeding the NO_3 diet significantly reduced butyrate proportion compared to the other dietary treatments ($P<0.01$). Across all diets, small Entodiniomorphs accounted for more than 90% of total protozoa present. Compared with CON treatment, total protozoal population (cells/ml) was decreased by 56% by O ($P=0.046$) with lesser population of small ($P=0.05$) and large Entodiniomorphs ($P<0.01$). Feeding NO_3 diet alone increased the population of large Entodiniomorphs by about 2.5 fold relative to the O treatment ($P<0.01$); however no protozoal differences were observed between NO_3 and NO_3+O-containing diets ($P=0.21$).

Table 2.3. Rumen fermentation and physiological characteristics of cattle on a mixed lucerne/barley diet (CON) with or without supplements of nitrate (NO_3), canola oil (O) or nitrate plus canola oil (NO_3+O).

	Diet[1]					P-value[2]		
Parameter	CON	NO_3	O	NO_3+O	SEM	Nitrate	Oil	Nitrate × oil
Final LW (kg)	715	711	712	705	4.78	0.21	0.30	0.74
DMI (kg/day)	6.64^{ab}	6.58^{ab}	6.86^{a}	6.36^{b}	0.05	<0.01	0.86	0.02
DMD (g/kg DM)	731^{a}	688^{b}	721^{a}	674^{b}	2.98	<0.01	0.60	0.02
MY (g CH_4/kg DMI)	23.2^{a}	21.2^{a}	21.8^{a}	17.4^{b}	0.62	<0.01	<0.01	0.09
DMP (g CH_4/d)	159.6	141.7	151.1	118.4	4.40	<0.01	<0.01	0.12
Rumen pH	6.2^{ab}	6.3^{a}	6.0^{b}	6.3^{a}	0.03	<0.01	0.17	0.04
Rumen ammonia (mgN/L)	121.8	166.1	127	196	6.46	<0.01	0.06	0.31
Total VFA (mmol/L)	104.2	99.5	106.5	100.1	1.61	0.20	0.93	0.93
Acetate (mol/100 mol)	62.3	67.4	59.1	64.1	0.42	<0.01	<0.01	0.69
Propionate (mol/100 mol)	16.1^{b}	15.2^{b}	19.1^{a}	15.8^{b}	0.28	<0.01	<0.01	0.03
Butyrate (mol/100 mol)	17.8^{a}	13.6^{c}	17.1^{ab}	16.2^{b}	0.33	<0.01	0.09	<0.01
Acetate:propionate	4.2	4.9	3.2	4.1	0.09	<0.01	<0.01	0.20
Total protozoa (x 10^5/ml)	20.9^{a}	14.0^{ab}	9.2^{b}	11.9^{ab}	1.83	0.48	0.10	0.02
Small Entodiniomorph	19.6^{a}	13.3^{ab}	8.4^{b}	11.0^{ab}	1.78	0.52	0.13	0.02
Large Entodiniomorph	0.9^{a}	1.3^{a}	0.5^{b}	0.7^{ab}	0.11	<0.01	0.78	<0.01
Small Holotrich	0.5	0.1	0.3	0.2	0.05	0.32	0.40	0.69
Large Holotrich	0.1	0.3	0.1	0.1	0.01	0.21	0.14	0.15

LW, live weight; DMI, DM intake; MY, methane yield; DMP, daily methane production; VFA, volatile fatty acids. [1]CON = control; NO_3 = CON + 20 g/kg as-fed basis calcium nitrate; O = CON + 50 g/kg as-fed basis canola oil; NO_3+O CON plus 20 g/kg as-fed basis calcium nitrate and 50 g/kg as-fed basis canola oil. [2]Nitrate represents the main effect of Nitrate (CON and O versus NO_3 and NO_3+O); Oil represents the main effect of Oil (CON and NO_3 versus O and NO_3+O); Nitrate × oil represents the interaction between main effects of Nitrate and Oil. [a,b,c]Mean values within a row with unlike superscript letters were significantly different for each dietary treatment ($P<0.05$).

2.4.3. Chemical parameters and ammonia-N kinetics in the rumen

Feeding NO_3 or NO_3+O diets increased daily N intake in cattle (P<0.01) compared to the other diets; and so increased the pool size of ruminal NH_3-N ($P < 0.01$) and tended to have greater ILR of ruminal NH_3-N (P=0.06). Adding NO_3 alone to the diet of cattle reduced the DM content of rumen digesta compared with the other dietary treatments (P<0.05; Table 2.4) whereas no effect was observed the NO_3+O treatment relative to CON (P=0.12) and O diets (P=0.65). Regarding rumen fluid, a greater rumen fluid outflow rate was observed in NO_3-containing diets (P=0.05) but rumen fluid MRT was not affected by NO_3 (P=0.57). Regarding rumen particles and DM, feeding nitrate increased rumen DM outflow rate (P=0.04). An additive effect between nitrate and oil (nitrate x oil, P=0.17) on reducing rumen particulate MRT (P=0.03) was observed.

Feeding oil-containing diets to cattle reduced the volume and MRT of the rumen fluid (P<0.01), as well as the pool size (P<0.01) and outflow rate of DM (P<0.01) from the rumen. Supplementation with canola oil reduced the pool size of rumen NH_3-N (P<0.01) and tended to reduce the proportion of ruminal NH_3-N utilized for microbial N (P=0.10) .There was no effect of the dietary treatment on microbial growth efficiency (P>0.05; Table 2.4).

Table 2.4. Rumen digesta and microbial dynamics of beef cattle fed a lucerne chaff/barley diet (CON) or supplemented with nitrate (NO_3), canola oil (O) or nitrate plus canola oil (NO_3+O).

	Diet[1]					P-value[2]		
Parameter	CON	NO_3	O	NO_3+O	SEM	Nitrate	Oil	Nitrate × oil
DM rumen digesta (g/kg)	120[a]	107[b]	137[a]	131[a]	1.90	<0.01	0.28	<0.01
Rumen fluid volume (l)	68.7	87.1	34.3	43.5	6.99	0.24	<0.01	0.42
Rumen fluid outflow rate (l/d)	54.7	78.1	48.3	69.0	5.22	0.05	0.28	0.50
Rumen DM pool size (kg)	11.2	7.3	5.1	4.3	1.11	0.35	<0.01	0.19
Rumen DM outflow rate (kg/d)	6.5	7.6	6.0	5.7	0.63	0.04	<0.01	0.70
Rumen fluid MRT (h)	34	27.7	16.9	15.3	3.00	0.57	<0.01	0.44
Rumen particulate MRT (h)	43.4	22.6	20.6	14.7	3.88	0.03	<0.01	0.17
Daily N intake (g N/d)	152[b]	174[a]	152[b]	177[a]	0.87	<0.01	0.36	0.04
Pool size of rumen NH_3-N (mmoles)	585	877	315	623	31.8	<0.01	<0.01	0.42
ILR of ruminal NH_3-N (g N/d)	165	220	149	200	13.3	0.06	0.52	0.11
Utilization of rumen NH_3-N for microbial N[3]	0.8	1.0	0.9	0.8	0.06	0.84	0.10	0.14
Microbial growth efficiency (g MicNAN/kg DOMI)	11.8	8.2	15.4	9.8	1.79	0.28	0.55	0.81

[1]CON = control; NO_3 = CON + 20 g/kg as-fed basis calcium nitrate; O = CON + 50 g/kg as-fed basis canola oil; NO_3+O CON with 20 g/kg as-fed basis calcium nitrate and 50 g/kg as-fed basis canola oil. [2]Nitrate represents the main effect of Nitrate (CON and O versus NO_3 and NO_3+O); Oil represents the main effect of Oil (CON and NO_3 versus O and NO_3+O); Nitrate × oil represents the interaction between main effects of Nitrate and Oil. [3]Proportion of microbial N derived from rumen ammonia. Calculated as Enrichment of ^{15}N in rumen bacteria/ Enrichment of ^{15}N in rumen ammonia.

MRT, mean retention time; N, nitrogen; ILR, irreversible loss rate; NH_3, ammonia; MicNAN, microbial non-ammonia N; DOMI, digestible organic matter intake. [a,b]Mean values within a row with unlike superscript letters were significantly different for each dietary treatment (P<0.05).

2.4.4. Rumen digesta flow

Flow of TD was less in cattle fed oil-containing diets ($P<0.05$; Table 2.5). However, no significant effects of the dietary treatments were observed for flow of DM, NAN or microbial NAN from the rumen of cattle ($P>0.05$). There was also no effect on the proportion of NAN leaving the rumen that was of microbial origin and no treatment differences in the mass of DMADR ($P>0.05$).

Table 2.5. Rumen flow of digesta and NAN as influenced by dietary treatment (control: CON; nitrate; NO_3; canola oil: O and nitrate plus canola oil: NO_3+O).

	Diet[2]					P-value[3]		
	CON	NO_3	O	NO_3+O	SEM	Nitrate	Oil	Nitrate × oil
Flow of TD (kg/d)	14.3	12.8	10.0	10.8	0.42	0.68	0.02	0.22
Flow of DM (kg/d)	2.0	2.0	3.3	2.5	0.34	0.43	0.38	0.43
DMADR (kg)	3.6	4.3	3.5	4.1	0.33	0.40	0.95	0.91
NAN flow, (g N/d)	64.4	58.3	72.5	54.7	5.40	0.17	0.95	0.45
Proportion of microbial NAN in TD[4]	0.58	0.52	0.62	0.58	0.04	0.26	0.58	0.26
Flow of microbial NAN (g micNAN/d)	36.4	30.4	40.1	31.2	2.30	0.10	0.58	0.75

NAN, non-ammonia nitrogen; TD, true digesta; DMADR, DM apparently digested in the rumen; N, nitrogen.

[2]CON = control; NO_3 = CON + 20 g/kg as-fed basis calcium nitrate; O = CON + 50g/kg as-fed basis canola oil; NO_3+O CON with 20g/kg as-fed basis calcium nitrate and 50 g/kg as-fed basis canola oil.

[3]Nitrate represents the main effect of Nitrate (CON and O versus NO_3 and NO_3+O); Oil represents the main effect of Oil (CON and NO_3 versus O and NO_3+O); Nitrate × oil represents the interaction between main effects of Nitrate and Oil.

[4]Calculated as Enrichment of ^{15}NAN in rumen TD/ Enrichment of ^{15}NAN in rumen microbes.

2.5. Discussion

The main objective of this experiment was to evaluate the effect of feeding nitrate and canola oil, alone or in combination, on enteric CH_4 production and nutrient supply to cattle, especially microbial NAN supply. It was shown that the effect of feeding nitrate plus canola oil in combination on reducing methanogenesis was more than additive and this was related to a shorter MRT of rumen particulate matter. There was, however, a negative effect of NO_3+O diet on DMD which may have contributed to the reduction in MY through reducing substrate availability as did the slight reduction in DMI.

2.5.1. Daily methane production and methane yield.

The antimethanogenic effect of nitrate has been well documented (Lee & Beauchemin, 2014). However, adding 20 g of nitrate/kg feed in this experiment had a less than expected effect on DMP. Assuming VFA proportions are unchanged, 1 mole of nitrate can be expected to reduce methanogenesis by 1 mole, so the inclusion of 20 g of NO_3/kg feed should have reduced methanogenesis by 0.323 mol or 5.17 g/kg DMI, however, the observed mitigation was only 39 % of that expected. Although this mitigation efficiency is lower than values obtained previously in beef cattle fed similar doses of dietary nitrate (Hulshof et al., 2012; Duthie et al., 2018), nitrate alone has been shown to be effective in multiple experiments from various forage to concentrate ratio diets (Hulshof et al., 2012; Velazco et al., 2014; Lee et al., 2017a; 2017b; Wang et al 2018).

In this experiment, the addition of nitrate to the diet of cattle reduced numbers of ciliate protozoa in the rumen, so indirectly contributing to the suppressive effect of nitrate on rumen methanogenesis and methanogens (Latham, Anderson, Pinchak & Nisbet, 2016) which live on and inside ciliate protozoa. Hydrogen (H_2) from oxidation of NADH generated by microbial fermentation of OM provides electrons that enable methanogens to reduce CO_2 to CH_4. The inhibition of methanogens in the rumen increases NADH availability and provides electrons for propionate production that compete with methanogenesis (Patra & Yu, 2012) as observed in the present experiment.

Canola oil is a source of long-chain fatty acids and the effectiveness in suppressing CH_4 is thought to be proportional to degree of unsaturation of the fatty acids (Giger-Reverdin, Morand-Fehr & Tran, 2003) as biohydrogenation of mono- and polyunsaturated fats provides an alternative hydrogen sink to reduction of carbon dioxide within the rumen.

However, there was no effect of adding canola oil alone in this experiment, as it has also been reported in cattle by Duthie et al. (2016). The CH_4 suppressing effect of dietary oils has been inconsistent in the literature, with Grainger and Beauchemin (2011) reporting that lipids reduced MY by approximately 2.4% per 10 g/kg DM fat added to the diet and Beauchemin, Kreuzer, O'Mara and McAllister (2008) reporting 5.6% CH_4 reduction for each 10 g of dietary fat/kg DM. An intermediate inhibitory effect was reported by Moate et al. (2011) who identified a 3.5% of CH4 reduction per each 10 g/kg DMI of fat.

The antiprotozoal effect of canola oil observed in this experiment has also been reported previously in cattle (Tesfa, 1993). Polyunsaturated fatty acids present in canola oil have a toxic effect on microorganisms involved in H_2 production and therefore reduce CH_4 (Doreau & Ferlay, 1995). Protozoa are H_2 producers and a reduction in their numbers in the rumen is usually associated with a decrease in DMP and MY (Nguyen & Hegarty, 2016). While the O diet reduced rumen protozoa by 56% compared to CON, the effect of the O diet on reducing methanogenesis was less substantial.

Our findings suggest that the effect of feeding the NO_3+O diet on reducing methanogenesis from beef cattle was more than additive. The 25% reduction in DMD observed in NO_3+O was in line with the findings of Guyader et al. (2015) and Popova et al. (2017). In combination, NO_3+O caused a reduction in MY that was greater than their individual effects (25% versus 8% and 6% respectively), which is consistent with canola oil and nitrate having different and complementary mechanisms for supressing methanogenesis (Guyader et al., 2015).

The antimethanogenic effect of feeding nitrate in combination with polyunsaturated lipids fed to dairy cattle has been shown to persist over time and to be long-term effective and safe not only for animals but also for humans, as nitrate and nitrate were not detected in milk or processed milk products (Moate et al., 2011; Guyader et al, 2016). In addition, Hegarty et al. (2016) reported that nitrates or nitrosamines were not detected in raw or cooked meat from beef cattle supplemented with 18 g nitrate/kg DM over 88 days in a feedlot.

Even though blood methaemoglobin was not determined in this experiment, there were no clinical signs of nitrate/nitrite toxicity in NO_3-fed steers, perhaps because a dietary adaptation period was included. Previous studies with cattle using similar doses of nitrate reported blood methaemoglobin levels below 26% suggesting no risk of toxicity to animals

(Guyader et al., 2015; Duthie et al., 2016). Additionally, because nitrate supplementation in ruminants has not been widely practiced due to the risk of NO_2 toxic effects, feeding nitrate in combination with canola oil, as well as including an adaptation period to the diet, appears to be a practical strategy that should enable the livestock sector to mitigate enteric CH_4 emissions. In particular, using this NO_3+O combination strategy could be a way of reducing the amounts of nitrate in the diet of ruminants, to achieve a mitigation target avoiding adverse effects on animals.

In terms of the effectiveness of the NO_3+O strategy in a different dietary or feeding system for beef cattle, Troy et al. (2015) reported that this strategy appears to be basal diet-dependent. These authors reported that nitrate in combination with rapeseed oil reduced CH_4 production in mixed forage and concentrate diet but no antimethanogenic effect was observed in high concentrate diets. However, Guyader et al. (2015; 2016) observed 30% reduction in CH_4 yield (gCH_4/kgDMI) using nitrate in combination with linseed oil in a 50:50 forage to concentrate diet. Other authors reported no effect of mixed forage and concentrate diets supplemented with nitrate and oil fed to cattle on methanogenesis (Doreau et al., 2017; Duthie et al., 2018).

2.5.2. Dry matter intake and digestibility

Although feed availability was restricted in this study, DMI was reduced by 4.2% in the NIT+O-fed steers, relative to the O diet. One previous experiment reported a reduction of 17% in DMI of dairy cows supplemented with 18 g nitrate/kg DM plus 35g linseed oil/kg DM but cattle were fed ad libitum (Guyader et al., 2016). Conversely, adding 21.5 g nitrate/kg DM plus 50 g rapeseed oil/kg DM did not reduce feed intake in beef cattle fed *ad libitum* (Troy et al., 2015). Intake depression in cattle fed diets supplemented with nitrate in combination with linseed oil has been related to a lower fiber digestibility (Guyader et al., 2016), as a negative correlation between voluntary intake and fiber digestibily exits through a lower passage rate of particles and rumen fill (Allen 1996). However, no significant effect of feeding the NO_3+O diet on rumen volume and rumen particulate MRT was observed in the present experiment.

In this experiment, there was no effect of feeding the NO_3 diet on DMI, in contrast to Lee et al. (2015) who observed a 15% reduction in DMI when beef cows were restrictively fed a diet including 25 g NO_3/kg DM. In addition, Klop et al. (2016) reported a reduction on

DMI in dairy cows fed 21 g nitrate /kg DM under restricted feeding regime. Feeding the OIL diet in this experiment increased the DMI of steers in agreement with previous experiments using canola oil in the diet of cattle (Johnson et al., 2002; Pinares-Patiño et al., 2016). However, Beauchemin & McGinn (2006) reported 21% reduction in DMI of beef cattle fed ad libitum a diet containing 46 g canola oil/kg DM.

The negative effect on DMD, of feeding nitrate as observed in this experiment is in agreement with Marais, Therion, Mackie, Kistner and Dennison (1988), although this has not always been reported (Olijhoek et al., 2016). The DMD reduction probably resulted from NO_2 having a direct toxic effect on rumen microbes, which alters the microbial population in cattle fed nitrate-containing diets. (Zhou, Yu & Meng, 2012)

A reduction in DMD has also been one of the most common findings associated with lipid supplementation in ruminants (Beauchemin & McGinn, 2006). Nevertheless, the lack of effect of feeding O on DMD observed in this experiment was in accordance with Brask et al. (2013), in dairy cows and may be related to the low inclusion of canola oil in the diet (50 g/kg DM). Patra (2013) suggested that when concentrations of lipids are below 70 g/kg DM, the lipids may not affect digestibility.

2.5.3. Rumen fermentation

Rumen fermentation as judged by total VFA concentration was not affected by dietary treatments in the current experiment. The greater proportion of butyrate detected in cattle fed the CON diet may be explained by the high number of protozoa in the rumen fluid which preferentially ferment OM to butyrate rather than acetate and propionate (Li, Davis, Nolan & Hegarty, 2012). Nitrate supplementation in the present experiment shifted rumen fermentation towards acetate and increased A:P, concurring with previous observations (Nolan et al., 2010). Nitrate has higher affinity for H_2 than does CO_2 and the reactions that generate propionate (Ungerfeld & Kohn, 2006) competing with CH_4 and propionate production.

In accordance with Machmüller, Ossowski and Kreuzer (2000) and Patra (2013), the proportion of acetate in VFA was unaffected by oil-inclusion but propionate percentage was significantly increased and so a reduction in A:P was observed. Adding canola oil to the diet of cattle may increase propionate proportion and reduce A:P (Pinares-Patiño et al., 2016), resulting in less hydrogen availability for CH_4 production, due to the net hydrogen

consumption in propionate synthesis (Newbold, de la Fuente, Belanche, Ramos-Morales & McEwan, 2015).

2.5.4. Rumen ammonia and microbial nitrogen outflow

In keeping with nitrate reduction to NO_2 and NH_3 in the rumen, ruminal NH_3-N concentration was increased by nitrate supplementation (NO_3 and NO_3+O treatments) which is in agreement with Hulshof et al. (2012). Rumen NH_3-N tended to be lost irreversibly at a greater rate in cattle fed nitrate-containing diets but no effects were observed on microbial growth efficiency (g MicNAN/kg DOMI) with all values at the lower end of reported ranges (Poppi & McLennan, 2010). Feeding nitrate has been shown to increase microbial N outflow in lambs fed a protein-deficient chaff (Nguyen, Barnett & Hegarty, 2016) and improved NH_3 incorporation into microbial protein in dairy cattle fed a low-protein diet (Wang et al., 2018). These results suggest that nitrate inclusion in the diet of ruminants may be more beneficial when N is limiting.

In this experiment, the concentration of NH_3-N in the rumen was slightly increased in the oil-containing diets in keeping with the significant reduction in the rumen protozoal population from the OIL treatment but in contrast with Beauchemin and McGinn (2006). In the same way, the significant decrease in ruminal protozoal population in cattle fed oil-containing diets is normally associated with increased microbial NAN outflow from the rumen (Ivan, Hidiroglou & Petit, 1991). However, no effects of the dietary treatment were observed in total and microbial NAN outflows in this experiment. Clearly, there are other factors affecting microbial NAN outflow and the lack of effect may have been a consequence of a smaller total rumen volume and hence total bacterial population in OIL-supplemented cattle.

The efficiency of microbial N synthesis is related to feeding level because higher DMI may increase ruminal passage rate of DM and the outflow of microbial protein (Barnett et al., 2012). In this experiment, oil-containing diets increased DMI and decreased rumen MRT but did not affect microbial growth in the rumen of cattle. The lack of effect of lipid supplementation on microbial growth (g MicNAN/kg DOMI) in this experiment has also been reported by Leupp, Lardy, Soto-Navarro, Bauer and Caton (2006).

2.5.5. Digesta kinetics

Methane yield is positively associated with DMD, MRT and rumen volume (Hegarty, 2004; Pinares-Patiño, Ebrahimi, et al., 2011; Goopy et al., 2014) and was observed for MY and DMD in cattle fed NO_3+O diet in this experiment The increase in the MY with a longer rumen MRT is probably caused by increasing the extent of digestion of structural carbohydrates, and a net reduction in CH_4 production per unit of feed digested (Pinares-Patiño, McEwan, et al., 2011). Our findings suggest that feeding the NO_3+O diet reduced DMP and MY which may be associated with the reduction in rumen MRT and rumen volume, caused by oil inclusion in the diet.

2.6. Conclusions

Together with existing publications, this experiment confirms that the effect of feeding dietary nitrate and canola oil in combination on reducing DMP and MY from beef cattle is greater than the sum of their individual effects. The combination of both dietary additives will allow high levels of CH_4 mitigation to be sustained with a lower dose of nitrate and therefore a reduced likelihood of NO_2 toxicity, which is a practical constraint to inclusion of nitrate in diets as a non-protein N source in ruminants. Moreover, the lower DMP and MY achieved with NO_3+O was consistent with a significant reduction in rumen MRT, although an adverse effect on DMD was observed. Reducing methanogenesis by the NO_3+O diet in this experiment did not improve microbial growth efficiency, the flow of MicNAN from the rumen or the utilization of rumen ammonia for microbial growth, suggesting that this strategy seems to be more useful in N-limiting diets if production benefits are also been sought from these CH_4 suppressive additives.

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Conflicts of interest

The authors declare no conflicts of interest.

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The effect of dietary nitrate and canola oil alone or in combination on fermentation, digesta kinetics and methane emissions from cattle.

Statement of authors' contribution

We, the Research 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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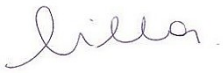

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Statement of originality

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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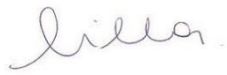

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Chapter 3. Dietary nitrate metabolism and enteric methane mitigation in sheep consuming a protein-deficient diet

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Dietary nitrate metabolism and enteric methane mitigation in sheep consuming a protein-deficient diet

3.1. Abstract

It was hypothesized that the inclusion of nitrate (NO_3) or cysteamine hydrochloride (CSH) in a protein deficient diet (4.8% crude protein; CP) would improve the productivity of sheep while reducing enteric methane (CH_4) emissions. A complete randomized designed experiment was conducted with yearling Merino sheep (n = 24) consuming a protein-deficient wheaten chaff control diet (CON) alone or supplemented with 1.8% nitrate (NO_3; DM basis), urea (Ur) or 80 mg cysteamine hydrochloride/kg live weight (CSH). Feed intake, CH_4 emissions, volatile fatty acids (VFA), digesta kinetics and NO_3, nitrite (NO_2) and urea concentrations in plasma, saliva and urine samples were measured. There was no dietary effect on animal performance or digesta kinetics ($P > 0.05$), but adding NO_3 to the CON diet reduced methane yield (MY) by 26% ($P = 0.01$). Nitrate supplementation increased blood MetHb, plasma NO_3 and NO_2 concentrations ($P < 0.05$), but there was no indication of NO_2 toxicity. Overall, salivary NO_3 concentration was greater than plasma NO_3 ($P < 0.05$), indicating that NO_3 was concentrated into saliva. Our results confirm the role of NO_3 as an effective additive to reduce CH_4 emissions, even in a highly deficient-protein diet and as a source of additional nitrogen (N) for microbial protein synthesis via N-recycling into saliva and the gut. The role of CSH as an additive in low quality diets for improving animal performance and reducing CH_4 emissions is still unclear.

Key words: ruminants, nitrate recycling, plasma, saliva, mean retention time, cysteamine hydrochloride

3.2. Introduction

Ruminant production based on low-quality forage is limited by the low availability of dietary nutrients to support efficient microbial growth and fermentation in the rumen (Leng, 1990). Improving the nutritive value of low-quality feeds in ruminant diets can considerable increase animal productivity, while maintaining or even decreasing CH_4 output (Gerber, 2013). Nitrate (NO_3) and cysteamine hydrochloride (CSH) have been shown to improve animal performance while reducing CH_4 emissions (Nguyen, Barnett & Hegarty, 2016; Sun et al., 2017).

Nitrate is an effective additive which can reduce enteric CH_4 production (Lee & Beauchemin, 2014) and can replace urea as a non-protein nitrogen (NPN) source, to improve the productivity of ruminants on low quality diets (Nolan, Hegarty, Hegarty, Godwin & Woodgate, 2010). Nitrate is firstly reduced to NO_2 and then to NH_3 by rumen microbes. However, if NO_2 accumulates in the rumen and enters into the bloodstream it may produce methaemoglobinaemia being the main limitation to the use of dietary NO_3 to mitigate enteric CH_4 production in ruminants (Hristov et al., 2013; Lund et al., 2014). More information is needed to fully understand the impact of NO_3 supplementation on whole-farm greenhouse gas (GHG) emissions, animal productivity and animal health (Hristov et al., 2013), as between-animal variation in blood methaemoglobin is considerable (Cockrum et al., 2010) and therefore NO_3 toxicity is unpredictable (Callaghan, Tomkins, Benu & Parker, 2014; Nolan, Godwin, de Raphélis-Soissan & Hegarty, 2016).

Cysteamine hydrochloride is a biological compound produced in the gastrointestinal tract and hypothalamus of all mammals, and it has been used as a dietary additive to improve growth and efficiency in a wide range of livestock species (Barnett & Hegarty, 2016). A reduction in enteric CH_4 has also been reported through the oral administration of CSH to sheep and cattle (Barnett & Hegarty, 2014; Sun et al., 2017). If dietary CSH improves growth and feed conversion ratio on low quality diets as reported for more digestible diets, it could provide a valuable means to improve productivity and reduce emissions intensity of ruminant production systems in the tropics. Little information is available about the performance of ruminants consuming low protein diets supplemented with CSH or NO_3, so an experiment was conducted to test whether the inclusion of NO_3 or CSH in a protein deficient chaff-based diet could improve the productivity of sheep while reducing their enteric CH_4 emissions.

3.3. Materials and methods

3.3.1. Animals and dietary treatments

The experiment was conducted over 57 days. All procedures involving animals were performed in accordance with the University of New England Animal Ethics Committee (AEC approval no. 16/068). Twenty-four female Merino sheep (27.5 ± 0.77 kg; 12 months of age) were selected according to liveweight (LW) and randomly assigned to one of four dietary treatments using stratified randomization. Sheep were housed in individual pens and acclimated to a control diet (CON) over two weeks prior to the start of the experiment with *ad libitum* access to feed and clean water. The CON diet was a wheaten chaff mixture (600 g wheaten chaff plus 200 g wheat grain). The second dietary treatment consisted of the CON diet with the inclusion of 1.8% nitrate/kg DM (NO_3, provided as 3.14% calcium nitrate, $5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway). The selected level of calcium nitrate included in the NO_3 diet was that which had previously achieved a measurable mitigation enteric CH_4 (Li et al., 2013; de Raphélis-Soissan et al., 2014). The NO_3 diet was offered as follows during acclimation: NO_3 at 0.9% of DM from Day 1 to Day 7, NO_3 at 1.4% of DM from Day 8 to Day 14 and NO_3 1.8% of DM from Day 14 onwards. The third dietary treatment consisted of the CON diet with the inclusion of urea (9.8 g urea/kg DM; Ur), which was gradually increased iso-nitrogenously to the NO_3 diet. The fourth treatment contained 80 mg/kg LW of cysteamine (CSH, cysteamine hydrochloride, > 95% purity, Tokyo Chemical Industry, Japan) dissolved in MiliQ® water each day, and mixed into the wheat grain portion of individual rations for each animal. The inclusion level of cysteamine-hydrocholide was based on previous studies in which 80 mg/kg LW improved sheep performance and reduced enteric CH_4 mitigation (Barnett & Hegarty, 2014). Urea and NO_3 straws were prepared weekly by spraying dissolved calcium nitrate or urea onto the wheaten chaff while mixing in a ribbon mixer. Animals were offered 800 g (fresh weight) of their experimental diet and were fed once a day at 0930 h. Offered feed and refusals were weighed and recorded daily throughout the experiment to estimate daily intake. Individual LW was recorded at the beginning of the experiment and then once weekly until the end of the experimental period.

3.3.2. Feed sampling and chemical analysis

Samples (200 g) of CON, NO_3, Ur diets, wheaten chaff and wheat grain were collected at weekly intervals after mixing and stored at -20°C. The wheaten chaff and wheat grain samples

were pooled and subsampled to analyse chemical composition at the NSW DPI Feed Quality Service, Wagga Wagga Agriculture Institute, using the methods described by AFIA (2014) (Table 3.1).

Table 3.1 Chemical composition of the wheaten chaff and wheat grain as ingredients of the control diet (% in dry matter; DM)

Component	Wheaten hay	Wheat grain
Dry matter (% as fed)	91	90
Dry matter digestibility	<39	87
Digestible organic matter	39	86
Water soluble Carbohydrate	<4	
Inorganic Ash	6	3
Organic Matter	94	97
Neutral detergent fibre	82	<10
Acid detergent fibre	52	<4
Crude protein	2	12.4
Crude fat	<1.1	1.4
Nitrite (ppm)	ND[A]	
Nitrate (ppm)	ND	
Metabolisable energyB (MJ/kg DM)	5	13.1
Gross energy (MJ/kg DM)	18	17.9

[A]Not detected

[B]Estimated metabolisable energy content of combined components in control diet was 7.03 MJ/kg DM

Feed and refusal samples were dried at 80˚C in a fan-forced oven to a constant weight to estimate DM percentage; then ground through a 1 mm sieve for subsequent analyses. Gross energy (GE) content in each diet (Table 3.2), feed refusal, faeces and in urine was determined by bomb calorimetry (Calorimeter C7000 with Cooling System C7002, IKA Werke®, Staufen Germany). Total nitrogen (N) concentration in feed offered and feed refusals were determined using a simultaneous carbon and nitrogen elemental analyzer (Leco® Corporation TruSpect, St Joseph. MI).

Table 3.2. Analysed nutrient composition (% in dry matter; DM) of the experimental diets fed to sheep. The control diet consisted in wheaten chaff plus wheat grain (CON) or supplemented with nitrate (18 g/kg DM; NO_3), urea (9.8 g/kg DM; Ur) or cysteamine hydrochloride (80 mg/kg liveweight; CSH

	CON	NO_3	Ur	CSH
Dry matter (%)	0.91	0.88	0.87	0.91
Nitrogen (%)	0.77	1.06	1.13	0.87
Gross Energy (MJ/kg DM)	16.76	16.54	16.74	17.01

3.3.3. Blood and saliva samples collection

To measure blood methaemoglobin (MetHb) concentration, blood samples of 6 mL were taken from each animal in all dietary treatments 1.5 h after morning feeding on Day 0 (0% NO_3 in DM), Day 7 (0.9% NO_3 in DM), Day 21 and Day 35 (1.8 % NO_3 in DM). Blood MetHb was measured within 30 min after sampling using an ABL 800 Flex blood gas analyser (Radiometer, Brønshøj, Denmark). Additionally, on Day 56 and Day 57 blood samples were taken before morning feeding (0 h), 1.5 h and 8 h after feeding in CON, NO_3 and Ur treatments to analyse NO_3, NO_2 and urea concentrations in plasma. Samples were taken from a jugular vein using lithium heparinized vacutainers (BD Vacutainer®, Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Plasma was separated via centrifugation for 15 min at 1050 x *g* at 4°C (Beckman® Coulter TJ-6, USA) and stored at -20°C until NO_3 and NO_2 concentration analyses.

Saliva samples (4 mL) were collected on Day 56 and Day 57 from sheep on CON, NO_3 and Ur diets before morning feeding (0 h), and 1.5 h and 8 h after feeding, by aspiration with a vacuum pump as described by Ortolani (1997). Two drops of concentrated sulfuric acid (H_2SO_4, 98%) were added and samples were centrifuged (10000*g* x 15 min) and stored at -20°C until subsequent analysis of NO_3 concentration. Plasma and saliva NO_3 and NO_2 concentration were determined by a continuous-flow analyser (San ++, Skalar, Breda, Netherlands) as described by de Raphélis-Soissan et al. (2017). Concentration of NO_2 in saliva was not determined because samples were acidified that causes a loss of NO_2. Urea concentrations in plasma and saliva were determined using a Dade Behring® Dimension Xpand Plus Integrated Chemistry Analyser (Ramsey, Minnesota, USA).

3.3.4. Rumen fluid sampling for volatile fatty acids, pH, ammonia and protozoa population

Filtered rumen fluid samples (20 mL) were collected on Day 35 using an esophageal tube with a brass filter tip at 3 h after morning feeding. Rumen pH was determined at the time of sample collection with a portable pH meter (Orion 230 Aplus, Thermo Scientific, USA) and samples were split into two subsamples for storage and further analysis. The first subsample of rumen fluid (15 mL) was acidified with 5 drops of concentrated H_2SO_4 and stored at -20°C for volatile fatty acids (VFA) and ammonia (NH_3-N) concentration as described by de Raphélis-Soissan et al. (2017). Rumen NH_3-N was determined by a continuous flow analyser (SAN ++, Skalar, Breda, Netherlands). Isocaproic acid was included as internal standard for VFA determination by gas chromatography using a Varian® CP-3800 chromatograph and Varian Star integration software as described by Nolan et al. (2010). A second subsample (4 mL) of rumen fluid was placed in a wide-neck McCartney bottle containing 16 mL of saline containing 4% v/v formaldehyde and stored at room temperature for visual enumeration of protozoa as described by Nguyen et al. (2016).

3.3.5. Methane production

Daily methane production (DMP; g CH_4/day) of each sheep was estimated over a 23 h period (Bird, Hegarty & Woodgate, 2008), with six sheep, being representative of all treatments, measured at one time. Sheep from each diet were placed randomly in one of 6 individual open circuit respiration chambers by 1000 h with their daily feed allocation and with water available *ad libitum*. Refused feed was measured at the end of the period (at 0900 h the following day). A continuous subsample of chamber gas (3 mL/min) was taken into a gas collection bag and CH_4 concentrations were determined using a photoacoustic infrared multigas analyser (Innova® Model 1312, Innova Airtech Instruments, Ballerup, Denmark). Daily CH_4 production was calculated as described by De Barbieri, Hegarty, Silveira and Oddy (2015). Energy loss through CH_4 was calculated assuming an energy content of 40.5 J/L CH_4 (Brouwer, 1965).

3.3.6. Digestibility of DM and digesta kinetics

After measurement of CH_4 production in respiration chambers, sheep were randomly allocated to metabolic cages in two different periods. Animals were dosed with non-digestible markers then placed in metabolism cages for assessment of digesta kinetics based on fecal marker excretion pattern. All animals were dosed with neutral detergent fibre (NDF) from oaten hay

mordanted with chromium (5 g of mordanted NDF, 299 mg Cr/sheep) and liquid-phase marker cobalt-EDTA (Co-EDTA, 4.5 g per sheep in 45 mL of MiliQ® water) from AVA Chemicals (Mumbai, India). The chromium mordanted NDF was prepared according to the procedure of Uden, Colucci and Van Soest (1980). Mordanted NDF was weighed in two portions of 2.5 g. Each portion was mixed with approximately 7.5 mL of MiliQ® water, introduced into a plastic tube (20 mm diameter x 70 mm length) and stored at -20 °C. Frozen pellets of mordanted NDF slurry were placed into a 30 cm length plastic tube and introduced in the rear of the sheep's buccal cavity, one at a time, before a flexible plastic rod was inserted into the tube to push the pellet into the oesophagus. Each animal received two frozen Cr-NDF pellets. A 40 mL warm water dose was given following each marker dose to help the ingestion and rinse the tube from residues. Co-EDTA solution was then administered as a single dose directly into the rumen via intubation.

After marker introduction, sheep were placed in metabolism cages and daily total collection of faeces, urine and feed refusals was conducted daily (2 periods × 6 animals/period over 6 days collection). Daily rations were offered at 1300 h on Day 1 and then at 0900 h and 1700 h on the subsequent days. Both offered and refused feed were weighed daily and a subsample of refusals was taken and stored in a plastic bag at -20°C for DM and GE determination. Faeces were collected and weighed at 1700 h, 2000 h, 2400 h on Day 1, at 0600 h, 0900 h, 1200 h and 1800 h on Day 2 and then at 0900 h and 1700 h on Day 3, Day 4, Day 5 and Day 6 until 0900 h on Day 7. From the homogenised fecal output of each interval, a 50 g fresh fecal subsample was taken, oven dried to constant weight at 65°C and ground though a 1-mm sieve for further analysis. Individual apparent digestibility of feed DM (DMD, %) was calculated for each animal using total DM intake and faecal DM output during the 6-day collection period. Analysis of Cr and Co concentrations was done by portable X-ray fluorescence spectroscopy using a Bruker Tracer III-V pXRF® with associated software (Bruker Corp., Billerica, Massachusetts, USA). Marker concentrations were determined using calibration curves published by Barnett et al. (2016). Studies of digesta DM and water kinetics were undertaken using non-linear curve fitting algorithms of WinSAAM (Aharoni, Brosh & Holzer, 1999). Digesta kinetics analysis was described previously in Goopy et al. (2014).

Urine collection and preservation was conducted according to the procedure of IAEA (1997). Urine samples were collected at 0500 h on Day 1, 0900 h and 0500 h on Day 2 and then daily

at 0900 h until Day 7. A 100 mL subsample was collected daily at 0900 h from Day 3 to Day 7. Urine samples were stored at -20 °C for subsequent analysis.

3.3.7. Statistical analyses

All data were analysed using R statistical software (R Core Team, 2016). A linear mixed effect model *lme* (Pinheiro, Bates, DebRoy & Sarkar, 2011) was used to analyse the effect of dietary treatments on sheep performance and CH_4 emissions. Diet was considered as a fixed effect. Period was considered as a random effect for data collected from metabolic cage and respiration chamber studies, where only six animals were studied per period. While in metabolic cages, one sheep on the NO_3 diet and one sheep on the Ur diet were removed because of low feed intakes. Data for DM intake (DMI) and LW were subject to repeated measures analysis using a linear mixed effect model, considering diet as a fixed effect and sheep ID as a random effect. Because of the lack of normality of data on plasma and salivary NO_3, NO_2 and urea, nonparametric statistical tests were used. For independent samples the Kruskal-Wallis test were employed, followed by the Mann-Whitney-Wilcoxon test for pairwise comparisons to detect differences between diets at each time. For dependent samples, Wilcoxon signed ranks tests were used for comparison of NO_3, NO_2 and urea concentrations in plasma and saliva within diets and across sampling time. Homogeneity of variance and normal distribution were tested using Q-Q plots and Shapiro-Wilk test. Data on blood MetHb concentrations and protozoa count were log-transformed before statistical analysis. Results were expressed as either mean ± s.e.m or median (95% lower-upper confidence intervals). *P*-values < 0.05 were regarded as significant and $P \leq 0.10$ as a tendency.

3.4. Results

3.4.1. Feed intake and liveweight

While sheep were in pens, no differences were observed in DMI due to dietary treatments (P = 0.79). Initial and final LW of sheep did not differ between treatments (P > 0.05; Table 3.3).

Table 3.3. The measurements in metabolic cages were made during a 6-day period of total collection of urine and faeces. LW, liveweight; DMI, dry matter intake; DMD, dry matter digestibility; DMP, daily methane production; MY, methane yield; GEI, gross energy intake. Means followed by a different letter are significantly different at P = 0.05.

Parameter	CON	NO_3	Ur	CSH	s.e.m	P-value
Initial LW (kg)	25.8	25.3	25.3	26.1	0.32	0.64
Final LW (kg)	25.2	26.0	23.9	25.3	0.34	0.23
DMI (g DMI/day) in pens	456	495	458	494	3.72	0.79
DMI (g DMI/day)[A]	415	492	458	434	23.9	0.73
N intake (g N/day)[A]	3.2a	5.2b	5.2b	3.8a	0.30	<0.01
Faecal N (g N/day)	2.5	2.7	2.7	2.6	0.12	0.96
Urinary N (g N/day)	3.0a	4.8b	4.3b	2.8a	0.24	<0.01
N balance (g N/day)	-2.3	-2.3	-1.8	-1.6	0.21	0.46
Apparent DMD (%)	48	47	44	42	1.45	0.30
DMP (g CH_4/d)	10.3	8.8	9.2	10.9	0.57	0.31
MY (g CH_4/kg DMI)	20.8b	15.4a	20.5b	21.3b	0.73	0.01
GEI (MJ/day)[A]	7.0	8.1	7.7	7.5	0.43	0.76
Energy loss in urine (MJ/day)	0.1	0.2	0.2	0.2	0.02	0.39
Energy loss in faeces (MJ/day)	3.8	4.5	4.2	4.3	0.22	0.68
Energy loss in methane (MJ/day)	0.6	0.5	0.5	0.6	0.03	0.34
GEI (MJ/day)[B]	9.5	11.1	9.0	9.9	0.44	0.30
Energy loss in methane/GEI[B] (%)	6.2b	4.4a	5.7b	6.1b	0.24	0.02
Metabolisable Energy (MJ/day)	2.5	2.9	2.7	2.4	0.17	0.72

[A]while in metabolic cages

[B]GEI while in respiration chambers

3.4.2. Dietary effect on N and energy utilization and methane emissions.

While sheep were in metabolic cages, there was no effect of the dietary treatment on DMI (P = 0.73; Table 3.3) or apparent DMD (P = 0.30). Nitrogen intake was greater in sheep fed diets supplemented with NO_3 or Ur than those fed CON or CSH diets (P < 0.01). Consequently, feeding NO_3 or Ur diets increased N excretion in urine (P < 0.01) whereas fecal N excretion was not affected by the dietary treatment (P = 0.96). No differences were observed in GE intake (GEI), GE loss as urine, faeces or CH_4 between dietary treatments (P > 0.05). Therefore there were no dietary effects on metabolisable energy (ME) intake between dietary treatments (P = 0.62; Table 3.3).

Sheep offered the NO_3 diet lost less energy through CH_4 as a proportion of GEI (P = 0.02; Table 3.3) compared to sheep offered the CON, Ur or CSH diets. Methane yield (MY, g CH_4/kg DMI) was significantly reduced in sheep fed the NO_3 diet (P = 0.01).

3.4.3. Rumen pH, ruminal volatile fatty acids and ammonia concentration, protozoa count and methane emissions.

Addition of NO_3 to the diet tended to increase ruminal pH (*P* = 0.05; Table 3.4) and both NO_3 and Ur diets significantly increased ruminal NH_3 concentrations (*P* < 0.01) relative to CON or CSH diets. There were no dietary effects on rumen VFA concentration (*P* = 0.45), acetate or propionate percentages (*P* > 0.05), but NO_3 tended to reduce butyrate percentage compared to CON or Ur diets (*P* = 0.05). In all the dietary treatments, small Entodiniomorphs accounted for 95% to 99% of total protozoa. Compared to CON-fed sheep, supplementation with NO_3 or CSH significantly reduced total protozoa number in the rumen fluid approximately 50% (*P* = 0.03 and *P* < 0.01 respectively) and tended to be lower in Ur-fed sheep (*P* = 0.07). Similarly, small Entodiniomorph concentrations were significantly reduced in sheep fed NO_3 or CSH (*P* = 0.003 and *P* = 0.026 respectively) and tended to be lower in Ur-fed sheep (*P* = 0.06). The numbers of large Entodiniomorphs were greater in sheep fed the Ur and CSH diets compared to the numbers in sheep fed the NO_3 diet (*P* = 0.02; *P* = 0.01 respectively; Table 3.4).

Table 3.4. Rumen fermentation characteristics and protozoal population of sheep fed a control diet of wheaten chaff plus wheat grain (CON) supplemented with nitrate (18 g/kg DM; NO_3), urea (9.8 g/kg DM; Ur) or cysteamine hydrochloride (80 mg/kg LW; CSH).

DM, dry matter; VFA, volatile fatty acid; A, acetate; P, propionate; NH_3, ammonia. Means followed by a different letter are significantly different at $P = 0.05$.

Parameter	CON	NO_3	Ur	CSH	s.e.m	*P* value
Ruminal pH	6.7a	6.9b	6.7a	6.7a	0.04	0.05
Total VFA concentration (mM)	58.1	50.7	60.2	59.1	2.16	0.45
Acetate (mol/100 mol)	70.7	75.0	68.4	70.9	1.04	0.17
Propionate (mol/100 mol)	22.1	20.7	24.7	22.7	0.92	0.57
Butyrate (mol/100 mol)	7.2a	4.2b	6.9a	6.4ab	0.42	0.05
A:P	3.2	3.6	2.8	3.1	0.16	0.44
Ruminal NH_3 (mM)	2.7a	9.7b	9.8b	4.5a	0.85	<0.01
Total protozoa (x 10^5/mL)	6.64b	3.46a	5.09a	3.07a	0.512	0.04
Small Entodiniomorph	6.59b	3.44a	4.90a	2.95a	0.507	0.04
Large Entodiniomorph	0.03ab	0.01a	0.13b	0.10b	0.019	0.04
Small Holotrich	0.01	0.01	0.04	0.02	0.009	0.60
Large Holotrich	0.01	0.00	0.02	0.00	0.004	0.22

3.4.4. Digesta and water kinetics.

Digesta kinetics as described by the mean retention time (MRT) in rumen solute (liquid) was not affected by the dietary treatment ($P > 0.05$; Table 3.5). There was a negative correlation between rumen MRT of the rumen solute fraction and DMI across all data (rumen MRT solute = 13.34 – 0.014 DMI, r = -0.58, $P = 0.01$). There were no significant effects on hindgut solute MRT ($P = 0.15$) or total solute MRT ($P = 0.08$). Greater DMI was associated with shorter total MRT across all data (solute MRT = 49.56 – 0.028 DMI, r = -0.72, $P < 0.001$). There was a tendency towards a negative correlation between the delay time for digesta to reach the hind gut and DMI across all data (delay time hindgut = 18.57 – 0.009 DMI, r = -0.46, $P = 0.06$).

Water kinetics as described by gastrointestinal transit time (time to first appearance of tracer in faeces) and whole gut MRT were not affected by the dietary treatment ($P > 0.0.5$; Table 3.5). There was a negative correlation between total MRT in the whole gut and DMI (Total MRT = 51.96 -0.028 DMI, r = -0.44, $P = 0.005$) and also between the intestinal transit time and DMI (Intestinal transit time = 24.99 – 0.025 DMI, $r^2 = -0.89$, $P < 0.001$).

Table 3.5. Water and digesta kinetic parameters of the rumen and intestine of sheep fed a control diet of wheaten chaff plus wheat grain (CON) supplemented with nitrate (18 g/kg DM; NO_3), urea (9.8 g/kg DM; Ur) or cysteamine hydrochloride (80 mg/kg LW; CSH).

DM, dry matter; MRT, mean retention time. Means followed by a different letter are significantly different at $P = 0.05$.

Parameter	CON	NO_3	Ur	CSH	s.e.m	*P*-value
Digesta kinetics						
Rumen solute MRT (h)	6.6	7.2	9.6	5.3	0.68	0.24
Hindgut solute MRT (h)	28.8	28.5	32.5	32.9	0.89	0.15
Total solute MRT (h)	35.4	35.7	42.1	38.2	1.04	0.08
Water kinetics						
Intestinal transit time (h)	14.6	13.1	14.1	12.7	0.60	0.15
Whole gut MRT (h)	37.3	38.8	42.4	39.4	1.18	0.52

3.4.5. Blood MetHb concentration

Concentrations of blood MetHb during weeks 1, 3 and 5 (5.4 ± 1.27% MetHb; $P < 0.001$) were significantly higher in NO_3-fed sheep than those in sheep supplemented with CON, CSH or Ur diets (1.0 ± 0.05% MetHb). Blood MetHb concentration changed from 4.1% to 6.9% over the 5 weeks in sheep supplemented with NO_3, although differences over time were non-significant ($P = 0.29$). Of six sheep in the NO_3 group, one sheep consistently had the highest concentration of blood MetHb while another sheep always had the lowest MetHb concentration of the group. The maximum value observed for blood MetHb concentration was 22.7% of total haemoglobin and no visual symptoms of NO_2 toxicity were observed.

3.4.6. Plasma and salivary nitrate, nitrite and urea concentrations

Overall, Kruskal-Wallis tests showed that plasma NO_3 and plasma NO_2 were different between diets ($P < 0.001$; Fig. 3.1A-3.1B). There was a large variation in plasma NO_3 within sheep fed the NO_3 diet (range 24 - 376 µM) which had greater plasma NO_3 at all sampling times compared to sheep fed the CON or Ur diets ($P < 0.05$). Within the NO_3 treatment, plasma NO_3 tended to be greater at 1.5 h after feeding than pre-feeding or at 8 h after feeding ($P = 0.09$; $P = 0.06$ respectively); and returned to pre-feeding levels after 8 h ($P = 0.44$). No differences were observed in plasma NO_3 across sampling time in sheep fed the CON or Ur diets ($P > 0.10$; Fig.3.1A).

Similarly to plasma NO_3, there was a large variation in plasma NO_2 among sheep fed the NO_3 diet (range 4 – 50 µM). Plasma NO_2 was less than plasma NO_3 in all dietary treatments and at all sampling times ($P < 0.05$), with sheep fed the NO_3 diet at 8 h after feeding being the exception ($P = 0.13$; Fig. 3.1B). Pre-feeding plasma NO_2 tended to be greater in sheep fed the NO_3 diet than in sheep fed the CON or Ur diets ($P = 0.06$). Within the NO_3 treatment, plasma NO_2 increased after feeding ($P = 0.03$), but levels returned to pre-feeding after 8 h ($P = 0.13$). Within sheep fed the CON diet, plasma NO_2 concentration was greater at pre-feeding compared to plasma NO_2 at 8 h after feeding ($P = 0.03$). In sheep fed the Ur diet plasma NO_2 concentration was greater at pre-feeding ($P = 0.02$) and at 1.5 h after feeding ($P = 0.03$) compared to plasma NO_2 at 8 h (Fig. 3.1B).

Concerning NO_3 concentration in saliva, the Kruskal-Wallis test indicated that salivary NO_3 significantly differed between diets ($P < 0.001$; Fig. 3.1A). Nitrate in saliva tended to be greater in sheep fed the NO_3 diet at pre-feeding and 1.5 h after feeding ($P < 0.10$). No differences were observed in salivary NO_3 between sheep fed the CON and Ur diets across sampling time ($P = 0.50$). Among sheep fed the NO_3 diet, salivary NO_3 tended to not return to pre-feeding levels after 8 h ($P = 0.06$). Sheep fed the CON diet had greater salivary NO_3 at 1.5 h after feeding compared to pre-feeding levels ($P = 0.02$) and returned to pre-feeding levels after 8 h ($P = 0.20$). No differences were observed in salivary NO_3 across sampling time in sheep fed the Ur diet ($P > 0.10$; Fig. 3.1A).

Salivary NO_3 was greater than plasma NO_3 in sheep fed the CON and Ur diets at all sampling times ($P < 0.05$; Fig. 3.1A). In particular, within sheep fed the NO_3 diet, plasma pre-feeding NO_3 concentrations tended to be greater than salivary NO_3 ($P = 0.06$), whereas NO_3 concentration in saliva tended to be greater than plasma NO_3 1.5 h after feeding ($P = 0.06$; Fig. 3.1A).

Plasma urea concentrations were significantly different between dietary treatments and ranged between 428 and 4355 µM ($P < 0.001$, Kruskal-Wallis; Fig. 3.1C). Feeding sheep with NO_3 or Ur diets increased plasma urea compared to sheep fed the CON diet at all sampling times ($P < 0.01$) while no differences were observed in plasma urea between sheep fed NO_3 or Ur diets ($P = 0.54$; Fig. 3.1C).

There was a significant effect of the dietary treatment on salivary urea ($P < 0.001$, Kruskal-Wallis; Fig. 3.1C). In general, salivary urea was greater in sheep fed NO_3 or Ur diets

($P < 0.01$) than in sheep fed the CON diet, except for salivary urea in sheep fed the CON and Ur diets 8 h after feeding ($P = 0.65$; Fig. 1C).

Urea concentrations pre-feeding and 1.5 h after feeding were greater in plasma than in saliva for sheep fed CON and NO_3 diets ($P < 0.05$; Fig. 3.1C), whereas no differences were observed 8 h after feeding ($P > 0.10$). Plasma urea was greater than salivary urea in sheep fed the Ur diet after 8 h ($P = 0.02$; Fig. 3.1C).

In considering total urinary excretion of urea after 6 d, no differences were observed between sheep fed CON (226 ± 33 mM), NO_3 (274 ± 54 mM) or Ur (394 ± 112 mM) diets ($P = 0.26$). However, total urinary excretion of NO_3 after 6 days was significantly greater ($P < 0.001$) in sheep fed the NO_3 diet (2.20 ± 0.35 mM) compared to sheep fed CON (0.8 ± 0.25 mM) or Ur diets (0.58 ± 0.26 mM).

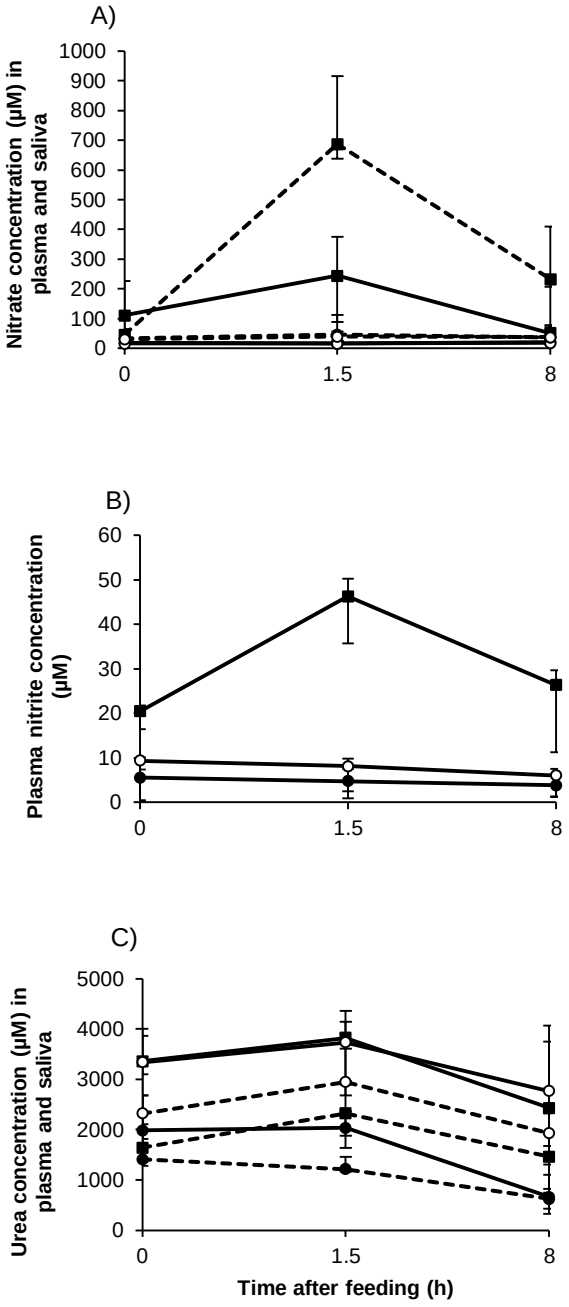

Figure 3.1. Median and 95% confidence intervals (CI) of nitrate[A] (A), nitrite (B) and urea (C) concentration (µM) in plasma (solid lines) or saliva (dashed lines) of sheep fed a control diet of wheaten chaff plus wheat grain (CON; solid circles) or supplemented with nitrate (18 g/kg DM; NO_3; solid squares) or urea (9.8 g/kg DM; Ur; open circles). Vertical bars indicate 95% CI. [A]Salivary NO_2 was not determined as samples were preserved with acid causing loss of NO_2.

3.5. Discussion

A significant effect of supplementing a protein-deficient diet with 1.8% NO_3 on MY reduction has been demonstrated in this experiment. However, supplementing with NO_3 or CSH did not improve animal productivity. Dietary NO_3 was shown to replace urea as an NPN source in a protein-deficient diet, and for the first time concentrating of blood NO_3 into saliva was demonstrated.

3.5.1. Effects of feeding nitrate on blood methaemoglobin

The occurrence of NO_2 toxicity in sheep after ingesting the NO_3 diet was evaluated by monitoring the occurrence of weakness, ataxia, trembling, breathing changes (no symptoms expressed) and by measuring blood MetHb concentrations weekly (Bruning-Fann & Kaneene, 1993). At the inclusion level in this experiment, NO_3 did not expose animals to NO_2 toxicity risk as evidenced by the low blood MetHb concentrations observed. Also, no clinical signs of NO_2 toxicity were observed in sheep in this experiment, although one animal in the NO_3 group exhibited blood MetHb concentration over 20% when dietary NO_3 was increased from 0.9% to 1.8% in DM. The low feed intake in sheep and the gradual adaptation of rumen microorganisms to the NO_3 diet may have contributed to the low blood MetHb concentrations observed on average in this experiment (Lee & Beauchemin, 2014; Benu et al., 2016).

3.5.2. Dietary effects on performance, N and energy utilisation and digesta kinetics

Regarding the nutritive value of the wheaten chaff plus wheat grain, the CON diet offered in this experiment provided 6.8 g CP per MJ of ME which was insufficient for yearling-sheep growth requirements (Freer, Dove & Nolan, 2007). Despite the NPN supplementation in the NO_3 and Ur treatments, these diets provided an average 8.9 g CP per MJ of ME. This is below the minimum CP content required for rumen microbial growth at which there is a risk of impaired ruminal fermentation (Freer et al., 2007).

Daily DMI was very low in all dietary treatments and sheep LW was stable throughout the experiment. The lack of effect of adding nitrate or urea on DMI, DMD and BWG in the current experiment was not expected and it is difficult to explain, since animals fed the control diet were N-deficient and low in rumen ammonia levels. Firstly, DMI in this experiment was very low and highly variable, which may have been related with the low quality of the chaff included

in all diets. Secondly, although rumen NH_3-N concentration were increased by adding nitrate or urea to the diet, there was no dietary effect (NO_3 or urea) on rumen fermentation as reflected by total VFA concentration.

In a similar experiment, Nguyen et al. (2016) found that lambs eating a protein deficient chaff supplemented with NO_3 had higher DMI and LW gain than lambs fed a non-supplemented diet, so a positive effect of NO_3 on DMI had been expected in this experiment. In addition, Wang et al. (2018) found that nitrate supplementation improved milk yield (kg/d) and ammonia incorporation into rumen microbial protein relative to urea in lactating dairy cows fed a low-protein diet However, in sheep fed N-adequate diets, previous studies reported no effect from replacing dietary urea with NO_3 on LW gain or DMI as was found in this experiment (Li, Davis, Nolan & Hegarty, 2012; Li et al., 2013). Nitrogen balance was slightly negative in all dietary treatments in the current experiment, suggesting that the low quality of the wheaten chaff (52% ADF) offered may have limited feed intake (1.86% of starting LW) and sheep growth.

The greater urinary N excretion in sheep fed the NO_3 or Ur diets relative to CON and CSH treatments may suggest an inadequate amount of fermentable carbohydrates relative to the level of protein provided by the NO_3 and Ur diets. However, NO_3-N excretion in urine increases in proportion to the amount of NO_3 ingested (Lee, Araujo, Koenig & Beauchemin, 2017) and with the increasing amount of dietary protein (Setchell & Williams, 1962). Similarly, the amount of urinary N is related to the CP content in the diet with increased urinary urea-N excretion when N intake increases (Nolan & Stachiw, 1979).

Replacing urea with NO_3 in the diet of sheep in this experiment did not modify total N excretion in urine but altered urinary N composition by increasing NO_3 relative to urea excretion. Total N excreted in urine did not differ when urea was replaced isonitrogenously with NO_3 in the diet in agreement with Van Zijderveld et al. (2011). Although not statistically significant, urea excretion was numerically greater in sheep fed the Ur diet whereas urinary NO_3 excretion was significantly greater in sheep fed the NO_3 diet relative to the other treatments. Considering that urinary urea is the major source of ammonia volatilised from manure (Lee et al., 2012), replacing urea with NO_3 in the diet of ruminants would contribute to decreased ammonia emissions from manure (Lee & Beauchemin, 2014).

Regarding the greater urinary NO_3 excretion in sheep fed the NO_3 diet relative to the other dietary treatments, Petersen et al. (2015) reported that NO_3 excreted in urine and faeces

from dairy cattle supplemented with different levels of dietary NO_3 were not significant sources of nitrous oxide (N_2O). They reported that N_2O was mainly exhaled from the animal, suggesting denitrification may occur in the rumen and in the oral cavity via NO_3 present in saliva. Recently, de Raphélis-Soissan et al. (2017) reported evidence of denitrification in the rumen and little N_2O emissions from the rumen when NO_3 was supplemented with 1% NO_3 in DM. However, the regulatory mechanism of denitrification pathways in the rumen and N_2O formation in the oral cavity require further investigation.

In considering energetic efficiency, the energy loss via CH_4 as percentage of GEI falls into the reported range of 5.5-6.5% (Johnson & Ward, 1996). Sheep fed the NO_3 diet had less energy loss through CH_4 as a percentage of GEI relative to CON sheep, concurring with Li et al. (2012). The lack of effect of feeding NO_3 on digesta kinetics characterised by MRT in the rumen of solute and particulate fraction was not expected, as a reduction in MRT in sheep fed a diet supplemented with NO_3 has been reported previously (Nolan et al., 2010; Nguyen et al., 2016). In the current experiment, across all dietary treatments, DMI was negatively related to MRT, a finding consistent with that reported by Nguyen et al. (2016) and also compatible with the understanding of rumen fill as the primary constraint to DMI in forage fed ruminants (Hogan, Kenney & Weston, 1987).

It has been shown that sheep fed a diet supplemented with CSH for 35 days improved animal performance (Barnett & Hegarty, 2016), but no beneficial effect of feeding CSH on animal growth was observed in this experiment. Previous findings reported no changes in liveweight of cattle fed a diet supplemented with CSH after 56 days (Sun et al., 2017). However, most studies using CSH as a feed additive have been conducted in animals fed diets containing adequate or high concentrations of N, and little other information is available on protein-deficient diets as provided in this experiment.

While no effects were observed in LW change and digesta kinetics in sheep fed CSH when compared to the CON diet, MRT of the rumen particulate fraction in sheep fed CSH was half the MRT in sheep fed the CON diet in accordance with Barnett et al. (2012) finding CSH accelerated digesta passage rate. It has been shown CSH administration to sheep produced a rapid depletion of somatostatin concentration and an indirect enhancement in animal growth (McLeod, Harmon, Schillo & Mitchell, 1995). However, under-nutrition can also alter the expression of somatostatin and the release of growth hormone (Henry, Rao, Tilbrook & Clarke, 2001) which may have moderated the effect of CSH on sheep performances in this experiment.

The findings related to lack of effect of CSH on digesta kinetics and animal growth appear to have been influenced by the fact the inadequate amount of N provided in the CSH diet was insufficient to support accelerated sheep growth.

3.5.3. Dietary effects on rumen fermentation and methane emission

Overall, ruminal NH_3 concentrations in sheep fed the NO_3, Ur and CSH diets were in the range sufficient for microbial growth (4.5 to 9.8 mM) (Satter & Slyter, 1974). In particular, ruminal NH_3 in sheep fed the CSH diet were suitable; whereas ruminal NH_3 concentration in sheep fed the CON diet was not adequate to support microbial fermentation and growth (Freer et al., 2007).

The increase in rumen pH from 6.7 to 6.9 observed on the NO_3 diet is of marginal biological significance and results were within a safe rumen pH range. The above statement is supported by the lack of effect of the dietary treatments on total VFA concentration, suggesting that there were no biologically important changes in rumen conditions. Additionally, the decreased molar percentage of butyrate decreased in sheep fed NO_3 compared with Ur is in agreement with Li et al. (2012) and with NO_3 reduction decreasing the availability of hydrogen in the rumen.

The presence of NO_2 in the rumen inhibits the growth of methanogens (Iwamoto, Asanuma & Hino, 2001) and protozoa (Sar et al., 2005) which are themselves hydrogen producers. However Nolan et al. (2010) and Li et al. (2012) reported no inhibitory effect of NO_3 on rumen protozoal population. Regardless of the negative effect of NO_3 diet on rumen protozoa observed in the current experiment there was no associated effect on DMP although MY (g CH_4/kg DMI) was significantly lower in NO_3-fed sheep compared to sheep fed the other diets. These results suggest that NO_3, as a feed additive to reduce enteric CH_4 emissions, is also effective in diets grossly deficient in CP. Nguyen et al. (2016) reported that dietary NO_3 reduced daily CH_4 production, but did not affect CH_4 yield (g/kg DMI) in lambs consuming a protein-deficient chaff. The effectiveness of NO_3 to reduce enteric CH_4 in sheep has been reported previously (Nolan et al., 2010; Li et al., 2012; de Raphélis-Soissan et al., 2014), but most studies have been conducted using N-adequate diets.

Adding CSH to the diet did not affect rumen fermentation parameters, but caused a 46% reduction in total rumen protozoa. Recent meta-analyses by Guyader et al. (2014) reported a linear relationship between CH_4 emissions and protozoal concentration and confirmed that

protozoa removal from the rumen can reduce enteric CH_4 emissions by 11% (Newbold, de la Fuente, Belanche, Ramos-Morales & McEwan, 2015). However, CH_4 production and MY were not reduced in sheep fed the CSH diet in this experiment which is in contrast to previous observations (Barnett & Hegarty, 2014; Sun et al., 2017).

3.5.4. Dietary effects on plasma, salivary and urinary nitrate metabolites and urea.

To evaluate enterosalivary NO_3-N or urea-N recycling, plasma and salivary NO_3 and urea concentrations were measured only in sheep fed the CON, NO_3 and Ur diets. The higher concentration of NO_3 in saliva compared to plasma in sheep over these treatments suggests that NO_3 was concentrated and recycled in the rumen via saliva and confirms the hypothesis suggested by Leng (2008). In humans, much of the circulating NO_3 is excreted in the urine and up to 25% is actively extracted by the salivary glands and concentrated via saliva (Spiegelhalder, Eisenbrand & Preussmann, 1976; Lundberg, Weitzberg & Gladwin, 2008). The results obtained in the present experiment show for the first time that NO_3-N recycling via saliva also occurs in ruminants, confirming the beneficial effect of NO_3 recycling to extend N availability over time to meet the requirements of rumen microorganisms (Leng, 2008). Additionally, the higher concentration of NO_3 in plasma than in saliva prior to feeding the NO_3 diet, may be a consequence of the recycling of NO_3 in the rumen, increasing the levels of recirculating NO_3 after feeding (Benu et al., 2016). Profiles of plasma NO_3 over time were consistent with previous observations (Sar et al., 2004; de Raphélis-Soissan et al., 2014).

Nitrite concentration in plasma was about 10-fold less than plasma NO_3 concentration in sheep fed the NO_3 diet. Nitrite remains stable for several hours in plasma, but in the whole blood is rapidly oxidised to NO_3 (Gladwin, 2004; Bryan, 2006), thus explaining the lower concentration of plasma NO_2 compared to plasma NO_3 and the return to pre-feeding levels after 8 h (Takahashi & Young, 1991; de Raphélis-Soissan et al., 2017).

The significant increase in plasma and salivary urea concentrations in sheep fed the NO_3 or Ur diets compared to sheep fed the CON diet suggests that both supplements increase salivary N supply for microbial protein synthesis. It has been reported that plasma and salivary urea concentrations are highly correlated (Hennessy & Nolan, 1988) so the decrease in plasma urea levels 8 h after feeding may explain why salivary urea returned to pre-feeding levels after 8 h. Additionally, in line with previous studies and in contrast to NO_3, plasma urea was greater

than salivary urea concentration (Hennessy & Nolan, 1988; Piccione, Foà, Bertolucci & Caola, 2006).

Apart from NO_3 recycling via saliva, absorbed dietary NO_3 was also excreted in urine, as indicated by the higher urinary excretion after 6 days in sheep fed the NO_3 diet compared sheep fed the CON or Ur diets. In relation to the concentration of urea excreted in urine after 6 days, the low results for all dietary treatments suggest that a large portion of dietary N metabolised within the animal was recycled into the gastrointestinal tract as a consequence of the low dietary N offered in this experiment (Cocimano & Leng, 1967; Van Soest, 1994).

The potential effect of salivary NO_3 excretion on denitrification and N_2O production in the oral cavity may offset the enteric CH_4 mitigation effect of dietary NO_3. However more research is needed to elucidate the mechanism behind N_2O production and emissions in ruminants fed diets supplemented with different levels of NO_3.

3.6. Conclusion

Our results suggest that the addition of 18 g NO_3/kg DM to a protein-deficient diet in ruminants had potential to mitigate CH_4 emissions without risking NO_2 toxicity, even when animals were only fed once daily. Interestingly, supplementing sheep diets with NO_3 as a NPN supply appears to be associated with additional N recycling into saliva and the gastrointestinal tract which is highly favourable when dietary N is limiting. Conversely, there was no positive role of CSH as an additive in protein deficient diets for improving animal performance and reducing CH_4 emissions within this experiment. Further studies investigating the mechanism and scope of bioactivity for CSH in ruminants consuming low quality diets are required.

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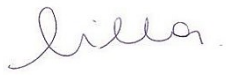

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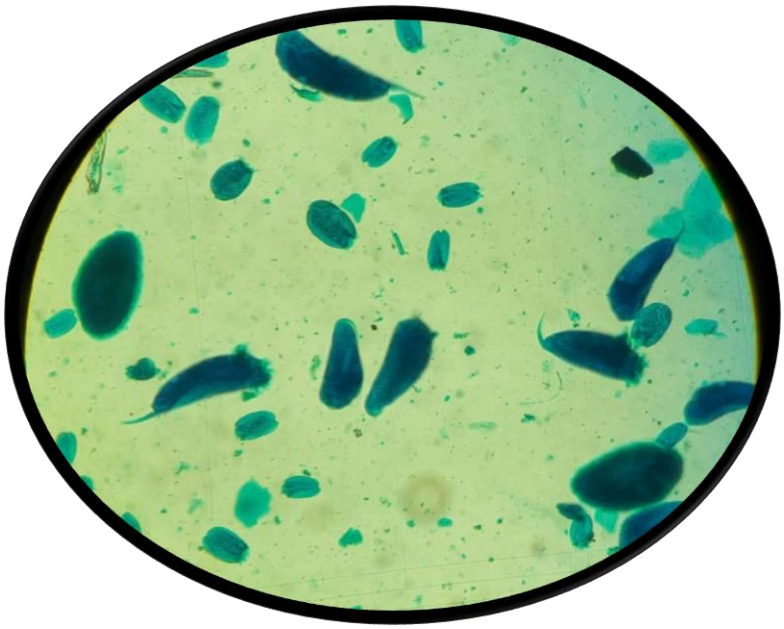
Chapter 4. Dietary nitrate and presence of protozoa increase nitrate and nitrite reduction in the rumen of sheep



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Dietary nitrate and presence of protozoa increase nitrate and nitrite reduction in the rumen of sheep

4.1. Abstract

Nitrate (NO_3) supplementation is an effective methane mitigation strategy for ruminants but may produce nitrite (NO_2) toxicity when NO_2 is absorbed from the rumen. It has been reported that rumen protozoa have greater ability for NO_3 and NO_2 reduction than bacteria. An *in vivo* experiment was conducted with faunated (FAU) and long term defaunated (DEF) sheep supplemented with 1.8% NO_3 in DM. The effects of rumen protozoa on concentrations of plasma and ruminal NO_3 and NO_2, blood methaemoglobin (MetHb), ruminal volatile fatty acid (VFA) and ruminal ammonia (NH_3) concentrations were investigated. Subsequently two *in vitro* experiments were conducted to determine the contribution of protozoa to NO_3 and NO_2 reduction rates in FAU and DEF whole rumen digesta (WRD) and its liquid (LIQ) and solid (SOL) fractions, incubated alone (CON) or with the addition of NO_3 or NO_2. The results from the *in vivo* experiment showed no differences in total VFA concentrations although ruminal ammonia (NH_3) was greater in FAU sheep ($p < 0.01$). Ruminal NO_3, NO_2 and plasma NO_2 concentration tended to be greater 1.5 h after feeding in FAU than in DEF sheep ($p < 0.10$). Ruminal and plasma NO_2 concentrations tended to increase in DEF sheep 3 h after feeding ($p < 0.06$). *In vitro* results showed that NO_3 reduction to NH_3 was stimulated by incoming NO_3 in both FAU and DEF relative to CON digesta ($p < 0.01$). However, adding NO_3 increased the rate of NO_2 accumulation in the SOL fraction of DEF relative to both fractions of FAU digesta ($p < 0.05$). Results observed *in vivo* and *in vitro* suggest that NO_3 and NO_2 are more rapidly metabolized in the presence of protozoa in the rumen. Defaunated sheep may have an increased risk of NO_2 poisoning due to NO_2 accumulation in the rumen.

Key words: defaunated, methaemoglobin, ruminants, disappearance rate

4.2. Introduction

Rumen protozoa can contribute up to 50% of the total bio-mass and by about 60% of the fermentation products in the rumen (Williams & Coleman, 1992; Newbold, de la Fuente, Belanche, Ramos-Morales & McEwan, 2015). However, protozoa are not essential to the ruminant survival and their role in rumen microbial ecosystem remains unclear. Feeding nitrate (NO_3) to ruminants is an effective strategy to mitigate CH_4 emissions (Lee & Beauchemin, 2014) while reducing protozoal number in the rumen (Morgavi, Forano, Martin & Newbold, 2010; Asanuma, Yokoyama & Hino, 2015). Protozoa appear to be important contributors to NO_3 reduction in the rumen as they have greater ability to reduce NO_3 and NO_2 than the bacteria fraction without causing NO_2 accumulation (Yoshida, Nakamura & Nakamura, 1982; Nakamura & Yoshida, 1991; Iwamoto, Asanuma & Hino, 2001; Lin, Schaefer, Guo, Ren & Meng, 2011). Dietary NO_3 is reduced to nitrite (NO_2) by rumen microorganisms and, depending on the reduction rate of NO_2 to NH_3, NO_3 can lead to NO_2 toxicity when NO_2 is absorbed (Lewis, 1951); making NO_2 toxicity the main drawback to NO_3 supplementation in ruminants (Nolan, Godwin, de Raphélis-Soissan & Hegarty, 2016). Blood methaemoglobin (MetHb) concentrations have been shown to increase in defaunated relative to faunated lambs suggesting a greater susceptibility to NO_2 toxicity in defaunated animals (Nguyen, Barnett & Hegarty, 2016). A review of the literature have also indicated that defaunation reduces ruminal total volatile fatty acid (VFA) and ammonia (NH_3) concentrations (Eugène, Archimede & Sauvant, 2004; Morgavi et al., 2010; Guyader et al., 2014; Newbold et al., 2015). The present experiments were conducted to quantify the effect of defaunation on *in vitro* rates of ruminal reduction of NO_3 and NO_2 as well as *in vivo* pH and concentrations of NH_3, VFA, NO_3 and NO_2 in the rumen. It was hypothesised that the absence of protozoa from the rumen of sheep may lead to higher NO_2 accumulation in the rumen and consequently a higher blood MetHb concentration due to a reduced ruminal reduction rate of NO_2.

4.3. Materials and methods

4.3.1. Animals, diet and experimental design

All procedures involving animals were performed in accordance with the Animal Ethics Committee of the University of New England (AEC17-090). Faunated (FAU; n = 4) and defaunated (DEF; n = 4) female sheep (82.3 ± 6.6 kg) were housed in individual pens for 37 days including 15 days of acclimation to a blended lucerne-oaten chaff diet supplemented with

NO_3 provided as calcium nitrate ($5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway). Feed was offered once daily as 2% of live weight/sheep at 0830 h. Sheep were fed the blended chaff supplemented with 0.9% NO_3 in DM during the first week and then 1.8% NO_3 in DM was offered until the end of the experiment. Water was freely available. Defaunation of sheep was performed as described in Nguyen, Nguyen, Bremner, and Hegarty (2018). Absence of rumen protozoa in DEF sheep was confirmed before conducting the experiment.

4.3.2. Blood methaemoglobin and plasma nitrate and nitrate concentrations

Blood samples were taken once weekly 1.5 h after feeding during the acclimation period to monitor for signs of NO_2 toxicity. Blood samples (10 mL) from each sheep were taken from the jugular vein using lithium heparinised vacutainers (BD Franklin Lakes, NJ. USA) on Day 17 before the morning feeding (0 h) and 1.5, 3 and 6 h after feeding. Blood MetHb concentration was determined in a subsample (2 mL) within 15 min of blood collection using a blood gas analyser (ABL 800 Flex, Radiometer, Brønshøj, Denmark). An 8mL subsample was centrifuged to separate plasma from red cells (1050*g* 4°C, 15 min; Beckman Coulter TJ-6, USA) and plasma was stored at -20°C. Nitrate and NO_2 concentrations in plasma were determined as described by de Raphélis-Soissan et al. (2017).

4.3.3. *In vivo* rumen volatile fatty acids, ammonia, nitrate and nitrite concentrations, pH and protozoa enumeration

Rumen fluid samples (30 mL) were collected on Day 17 before morning feeding (0 h) and 1.5, 3 and 6 h after feeding, using an esophageal tube with a brass filter tip. Rumen pH was determined at the time of sample collection with a portable pH meter (Orion 230 Aplus, Thermo Scientific, USA). Rumen fluid was then split into subsamples. One 10 mL subsample was acidified with 0.25 mL of concentrated sulfuric acid (98%; H_2SO_4) for subsequent analyses of ruminal VFA and NH_3 concentrations. Another 10 mL subsample was preserved 0.5 mL 37% formaldehyde for NO_3 and NO_2 determinations as described by de Raphélis-Soissan et al. (2017). These samples were stored at -20°C. A 4 mL subsample was preserved in 16 mL of 4% formaldehyde saline and stored at room temperature for protozoa enumeration. Volatile fatty acids were determined by gas chromatography according to Nolan, Hegarty, Hegarty, Godwin and Woodgate (2010). Protozoa were stained with brilliant green as described by Nguyen &

Hegarty (2016) prior to enumeration in a Fuchs-Rosenthal optical counting chamber (0.0625 mm^2, 0.2 mm depth) using a technique adapted from Dehority (1984).

4.3.4. *In vitro* (1). Determination for NO_3 and NO_2 reduction rates in whole rumen digesta

Whole rumen digesta samples (WRD; 70 mL) were collected 24 h post-feeding on Day 36 by oesophageal intubation from all sheep. Rumen digesta was kept at 39°C and transported to a 37°C temperature controlled room and subsampled (12 x 4.5 mL) into capped 15 mL N_2-flushed tubes. These were pre-buffered with 0.5 mL of 50 mM degassed phosphate buffer (sodium phosphate dibasic and sodium phosphate monobasic; pH 6.5. To prevent NH_3 liberated into the incubation from being incorporated into amino acids by rumen bacteria, 1 mM hydrazine sulfate and 30 μg/mL chloramphenicol were included to inhibit microbial N metabolism (Broderick, 1987). Tubes were allocated according to 2 x 2 factorial design (protozoa status either FAU or DEF; addition of NO_3 or addition of NO_2). The first treatment (CON) included four subsamples (4.5 mL) of WRD plus 0.5 mL buffer including the inhibitors. The second treatment (NO_3) consisted of four subsamples (4.5 mL) of WRD plus 0.5 mL NO_3 buffer containing 5 mM NO_3 with inhibitors. The third treatment (NO_2) involved four subsamples (4.5 mL) of WRD plus 0.5 mL NO_2 buffer containing 5 mM NO_2 and inhibitors. Nitrate and NO_2 concentrations in the buffer were selected according to previous *in vitro* experiments where no adverse effect on the growth of rumen microorganisms was observed (Iwamoto, Asanuma & Hino, 1999). All tubes were inverted for mixing and two subsamples of each treatment were immediately submerged into ice (0 min) and the other two subsamples were placed into a 39°C water bath for 15 min before removal and ice immersion for 15 min. An aliquot (1 mL) of the cooled 0 and 15 min incubations from all treatments was further subsampled into 2 mL microfuge tubes with one drop of concentrated sulfuric acid (H_2SO_4) added for later rumen NH_3 determinations. All samples were immediately stored at -20°C for further analyses.

4.3.5. *In vitro* (2). Measurement of NO_3 and NO_2 reduction rates in solid and liquid fractions of rumen digesta

Rumen digesta (70 mL) was collected at 24 h post-feeding on Day 37 by oesophageal intubation from all sheep. Rumen digesta was transported to a 37°C temperature controlled room and split into two 35 mL subsamples. These 35 mL subsamples were centrifuged in pre-warmed centrifuge tubes (37 °C) at 150*g* for 3 min (Beckman Coulter Allegra® X-12R Centrifuge) to precipitate ciliate protozoa following the method of Ogimoto and Imai (1981). WRD and resultant solid (SOL) and liquid (LIQ) fraction volumes were recorded. From each 35 mL centrifuged rumen digesta subsample, 12 aliquots (2.25 mL) of each LIQ and SOL fraction (vortexed after LIQ samples were taken) of rumen digesta were further transferred to 15 mL capped N_2-flushed tubes containing 0.25 ml of degassed buffer with inhibitors. This followed the same procedures and used the same concentrations of NO_3 and NO_2 in each treatment as those in *in vitro* experiment 1. Tubes were allocated according to a 2 x 2 x 2 factorial design (protozoa status either FAU or DEF; rumen digesta fraction either LIQ or SOL; addition of NO_3 or addition of NO_2). Treatments consisted of LIQ or SOL rumen digesta fractions incubated alone (CON) or with the addition of NO_3 (NO_3), or NO_2 (NO_2).

Concentration of NH_3 was determined on a modified Berthelot reaction as described by de Raphélis-Soissan et al. (2017). Concentrations of NO_3 and NO_2 in rumen digesta samples deproteinised by adding zinc sulphate to give a final concentration of 15g/L were determined by Griess reaction, as described by Moshage, Kok, Huizenga and Jansen (1995). A continuous flow analyser (SAN+ +, Skalar, Breda, Netherlands) was used for NH_3, NO_3 and NO_2 determination.

4.3.6. Calculations and statistical analysis

All *in vivo* variables were analysed in a complete randomized design using R (R Core Team, 2016). Data on diurnal rumen pH, NH_3, VFA, plasma NO_3 and NO_2 and blood MetHb concentrations were analysed as repeated measures using a mixed model *lme* (Pinheiro, Bates, DebRoy & Sarkar, 2011). Protozoa status in the rumen digesta (FAU or DEF) was considered as a fixed effect and sheep as a random effect. Data on blood MetHb concentrations was log transformed before analysis. Data from analytical replicates were averaged. Because of lack of normality of data on *in vivo* NO_3 and NO_2 concentrations in the rumen digesta, non-parametric tests were used. For independent samples the Kruskal-Wallis test was employed, followed by the Mann-Whitney-Wilcoxon test for pairwise comparisons to detect differences between FAU

and DEF sheep at each time. For dependent samples, Wilcoxon signed rank tests were used for comparison of NO_3 and NO_2 concentrations in rumen digesta within FAU and DEF treatments and across sampling times. Nitrate and NO_2 disappearance, NO_2 accumulation and NH_3 accumulation rates were determined via differences in the measured concentrations in rumen digesta samples collected at 0 min and after 15 min incubation *in vitro*. Nitrate and NO_2 disappearance rates and NH_3 accumulation rates were calculated in treatments CON and NO_3 whereas NO_2 and NH_3 accumulation rates were calculated in treatments CON and NO_2. Nitrate and NO_2 disappearance rates and NO_2 and NH_3 accumulation rates in WRD, LIQ and SOL fractions of rumen digesta were subject to an ANOVA using linear mixed models. For the first *in vitro* experiment, ANOVA was based on a 2 x 2 factorial design with factors being protozoa status (FAU or DEF); treatment (CON, NO_3) or (CON NO_2) and protozoa x treatment interaction. Protozoa and treatment were considered as fixed effects and donor sheep as a random effect. For the second *in vitro* experiment an ANOVA based on 2 x 2 x 2 factorial design was used with factors being protozoa status (FAU or DEF); treatment (CON, NO_3) or (CON, NO_2) and rumen digesta fraction (LIQ and SOL) and interactions. Protozoa, treatment and rumen digesta fraction were considered as fixed effects and the donor sheep as a random effect. Means were analysed using the least-square means procedure. Results were expressed as either mean ± SEM unless specified median and interquartile range (IQR) or median ± 90% lower-upper confidence intervals in graphs. Coefficient of variation (CV) was specified for plasma NO_2 and NO_3 concentrations. Statistical significance of differences was taken as *p*-values < 0.05, and trends were considered when $0.05 < p < 0.10$.

4.4. Results and discussion

The principal objective of this research was to investigate the effect of presence or absence of rumen protozoa, *in vivo* and *in vitro,* to NO_3 metabolism in the rumen and the risk of NO_2 poisoning in FAU and DEF sheep adapted to dietary NO_3. Ruminants are able to survive without rumen protozoa but the protozoa's significance in ruminant nutrition remains unclear. Removing protozoa from the rumen can improve the performance of animals on low protein diets (Eugène et al., 2004). However, it appears that protozoa are also important contributors to the bacterial reduction of NO_3 to NH_3 in the rumen (Yoshida et al., 1982; Lin et al., 2011), reducing the risk of NO_2 toxicity (Yoshida et al., 1982). The key observations of the current experiments were that the presence of NO_3 and protozoa stimulated NO_3 reduction to NH_3 and that NO_2 accumulated in the rumen digesta when protozoa were absent.

4.4.1. Rumen metabolites

Rumen NH_3 concentration in DEF sheep was lower than in FAU sheep pre-feeding ($p < 0.01$) and tended to be lower 1.5 h post-feeding ($p = 0.05$; Table 4.1). The decrease in rumen NH_3 concentrations, as observed in this experiment, is probably the most consistent effect of defaunation reported in the literature (Eugène et al., 2004; Morgavi, Martin, Jouany & Ranilla, 2012; Nguyen et al., 2018), suggesting greater recycling of nitrogen within the rumen in FAU sheep as a consequence of bacterial predation and subsequent protozoal lysis (Williams & Coleman, 1992; Jouany, 1996). Total VFA concentration in the rumen fluid was not affected by defaunation in this experiment ($p = 0.24$) in agreement with previous findings (Nguyen et al., 2018). However, a reduced concentration of total VFA in the rumen following defaunation has often been reported (Williams & Coleman, 1992; Eugène et al., 2004; Newbold et al., 2015). Changes in the molar proportions of the VFA in defaunated ruminants appear to be not directly attributable to the protozoa fraction but arise indirectly as a result of the increased concentration of bacterial that accompanies a reduction in protozoal numbers (Williams & Coleman, 1992). Numbers of protozoa 1.5 h after feeding in in FAU sheep were 12.4 (±1.71) $x10^5$ cells/mL. No protozoa were counted in DEF sheep 1.5 h after feeding.

Table 4.1. The effect of the presence (FAU) or absence (DEF) of protozoa (P) on *in vivo* rumen digesta parameters in sheep sampled at 0, 1.5, 3 or 6 h after feeding lucerne chaff supplemented with 1.8% NO_3 in DM

	FAU (n = 4)				DEF (n = 4)				*p*-value		
	0 h	1.5 h	3 h	6 h	0 h	1.5 h	3 h	6 h	SEM	P	Time
Rumen pH	7.11^{a}	7.06^{a}	7.05^{a}	6.85^{bc}	7.25^{a}	7.10^{ab}	7.10^{ab}	6.88^{c}	0.025	0.14	<0.01
Rumen NH_3 (mM)	10.98^{b}	18.40^{a}	15.89^{c}	12.81^{bc}	7.15^{d}	13.60^{b}	13.83^{b}	10.25^{bd}	0.661	0.03	<0.01
Total VFA concentration (mM)	72.79^{c}	83.04^{abc}	96.10^{ab}	92.65^{ab}	55.46^{cd}	84.28^{abc}	88.87^{abc}	90.16^{abc}	2.707	0.24	<0.01
Acetate (mol/100 mol)†	71.45^{d}	76.99^{ab}	75.73^{b}	75.20^{b}	79.28^{abc}	81.85^{ac}	81.03^{ac}	80.54^{c}	0.624	<0.01	<0.01
Propionate (mol/100 mol)	16.48^{b}	17.10^{ab}	17.60^{a}	17.65^{a}	12.35^{d}	12.71^{cd}	13.52^{c}	13.64^{c}	0.373	<0.01	<0.01
Butyrate (mol/100 mol)‡	8.09^{a}	3.84^{bc}	4.18^{bc}	4.85^{bc}	5.37^{ab}	2.99^{c}	3.31^{c}	3.88^{c}	0.320	0.07	<0.01
A:P§	4.34^{b}	4.51^{b}	4.31^{b}	4.26^{b}	6.45^{a}	6.47^{a}	6.01^{ac}	5.91^{c}	0.167	<0.01	<0.01
Total protozoa (x10_5 cells/mL)	na	12.40	na	na	na	0	na	na	1.710		

† Interaction term P x time (p = 0.05)

‡ Interaction term P x time (p = 0.08)

§ Interaction term P x time (p = 0.09)

na= data not available

Defaunated sheep had a greater acetate proportion ($p < 0.01$; Table 4.1) and a reduced propionate proportion in VFA than FAU sheep ($p < 0.01$) so acetate:propionate ratio (A:P) was greater in DEF sheep at all sampling times ($p < 0.01$). An increase in acetate proportion in DEF relative to FAU sheep observed in this experiment is consistent with previous findings (Hegarty, Bird, Vanselow & Woodgate, 2008; Newbold et al., 2015). However, the ruminal proportion of propionate was lower in DEF relative to FAU sheep in agreement with (Guyader et al., 2014) but in contrast to earlier findings where propionate proportion was increased or not affected by defaunation (Eugène et al., 2004; Newbold et al., 2015). The reduction in the molar proportion of butyrate in DEF sheep ($p = 0.01$) is in accordance with previous findings (Eugène et al., 2004; Newbold et al., 2015). In addition, there was no significant difference in ruminal pH between FAU and DEF sheep ($p = 0.14$) and pH values observed in this experiment were close enough to neutrality as to not adversely affect fibre fermentation.

Although presence of protozoa had no significant effect on total VFA concentration, DEF sheep had greater acetate but lower propionate and butyrate proportions than FAU sheep. Rumen protozoa are hydrogen (H_2) producers and synthesize mainly acetate and butyrate rather than propionate (Williams and Coleman). Therefore an increase in propionate proportion in the VFA is expected after defaunation, although contradictory results have been reported in the literature (Williams and Coleman, 1992; Newbold et al., 2015). In addition, removal of methanogens associated with protozoa in DEF sheep may have stimulated reductive acetogenesis which involves the reduction of CO_2 by H_2 to acetate (Ungerfeld 2013).

The inconsistent effects of defaunation within the literature on rumen VFA concentration and molar proportions may suggest variable effects on ruminal bacteria. Elimination of the ciliate protozoa increases the number of bacteria, which induces changes in digestion and fermentation due to changes in bacterial composition (Belanche, de la Fuente, Pinloche, Newbold & Balcells, 2012; Newbold et al., 2015).

There was substantial diurnal and between-sheep variation in ruminal concentrations of NO_3 (median = 0.06 mM; IQR = 0.03, 1.33 mM) and NO_2 (median = 0.01 mM; IQR = 0.01, 1.08 mM) in FAU sheep (Figure 4.1A - 4.1C) but this was not observed for ruminal NH_3 concentrations *in vivo* (Table 4.1) or *in vitro* (Tables 4.2 – 4.5) suggesting that the reduction and/or absorption of NO_3 and NO_2 are more variable than the much larger NH_3 flux. Regarding DEF sheep, ruminal concentrations of NO_3 (median = 0.16 mM; IQR = 0.05, 1.74 mM) and NO_2 (median = 0.05 mM; IQR = 0.01, 1.86 mM) were also highly variable (Figure 4.1B and

4.1D). One FAU sheep exhibited ruminal NO_3 and NO_2 concentrations about 2-fold greater than the mean of the group immediately (1.5 h) and 6 h after ingesting dietary NO_3. Similarly, one DEF sheep that showed ruminal NO_3 and NO_2 concentrations between 2 and 3-fold greater than the mean of the group on each sampling time (Figure 4).

Table 4.2. Nitrate (NO_3) disappearance and nitrite (NO_2) and ammonia (NH_3) accumulation rates measured *in vitro* in 4.5 mL of whole rumen digesta, as influenced by presence (FAU) or absence (DEF) of rumen protozoa (P), incubated for 15 min with 50 mM phosphate buffer containing 1 mM hydrazine sulfate and 30 µg/mL chloramphenicol, with the absence (CON) or addition (NO_3) of 5 mM NO_3. Analysed initial concentrations of NO_3, NO_2 and NH_3 in rumen digesta at 0 min are also shown.

	DEF		FAU			*p*-value		
	CON	NO_3	CON	NO_3	SEM	P	NO_3	P x NO_3
Initial concentrations, µmol/L								
NO_3	6	370	57	498	43.2	0.84	<0.01	<0.01
NO_2	56	220	68	469	49.0	0.06	0.03	0.11
NH_3	8283	6681	8190	7474	341.4	0.61	0.11	0.52
Rates, µmol/L/min								
NO_3 disappearance	0.09	15.70	2.57	17.48	2.317	0.44	<0.01	0.90
NO_2 accumulation	0.13	60.82	0.20	24.93	8.717	0.21	0.01	0.21
NH_3 accumulation	18.52	51.66	39.21	61.04	7.979	0.28	0.07	0.68

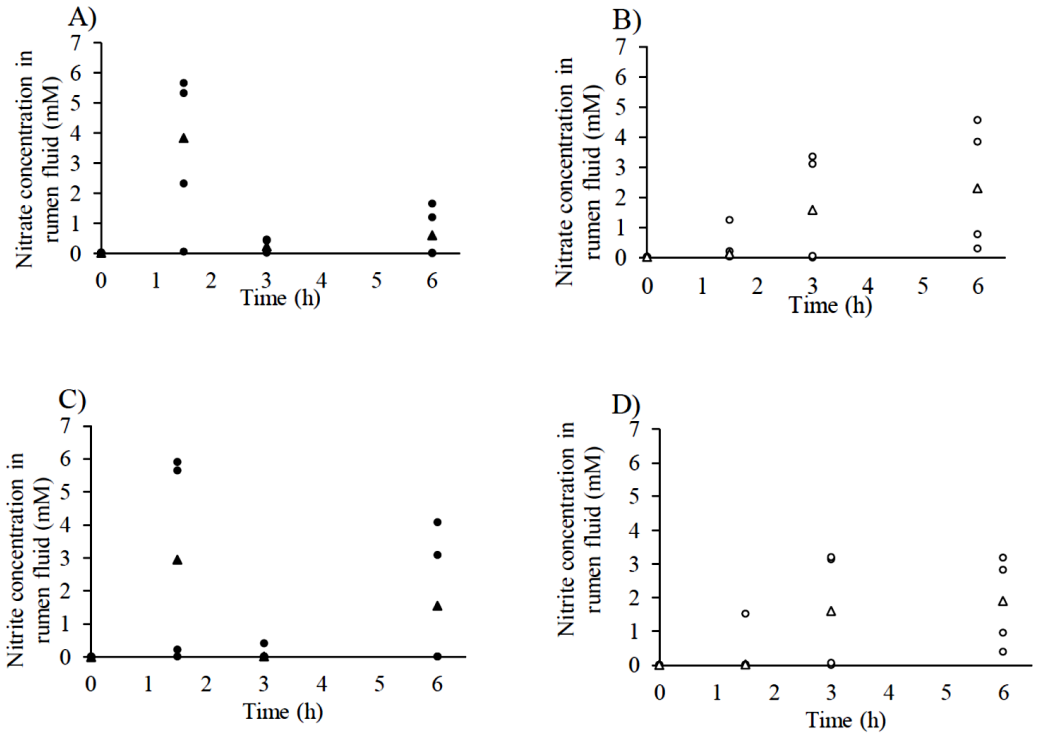

Figure 4.1. Nitrate (A&B) and nitrite (C&D) concentrations (mM) in rumen fluid of individual faunated (●; ▲) and defaunated (○; Δ) sheep at pre-feeding (0 h) and after feeding (3 h and 6 h) a diet containing 1.8% NO_3 in DM. Triangles indicate median value of each group of sheep. Circles indicate individual values for each sheep.

The large variation among sheep in rumen concentrations of NO_2 and NO_3 observed in this research suggests differences in ruminal NO_3 and NO_2 metabolism between animals (de Raphélis-Soissan et al., 2017). Individual animals differ considerably in their tolerance to supplementary NO_3, partially explained by differences in the microbial eco-system and hepatic gene expression (Cockrum et al., 2010) and possibly due to differences in the production of nitric oxide from NO_2 in the rumen, as nitric oxide has profound physiological effects in ruminants (Nolan et al., 2016).

Table 4.3. Effect of rumen protozoa (P) on *in vitro* nitrite (NO_2) disappearance and ammonia (NH_3) accumulation rates measured in 4.5 mL of whole rumen digesta from faunated (FAU) or defaunated (DEF) sheep, incubated for 15 min with 50 mM phosphate buffer containing 1 mM hydrazine sulfate and 30 µg/mL chloramphenicol with the absence (CON) or addition (NO_2) of 5 mM of NO_2. Analysed initial concentrations of NO_3, NO_2 and NH_3 in rumen digesta at 0 min are also shown.

	DEF		FAU			*p*-value		
	CON	NO_2	CON	NO_2	SEM	P	NO_2	P x NO_2
Initial concentrations, µmol/L								
NO_2	56	133	68	111	12.6	0.36	0.97	0.79
NH_3	8283	8540	8190	8552	292.1	0.95	0.64	0.94
Rates, µmol/L/min								
NO_2 disappearance	0.13	4.11	0.20	1.45	0.691	0.32	0.06	0.30
NH_3 accumulation	18.52	35.33	39.21	38.76	6.687	0.41	0.57	0.55

There was a very low NO_3 concentration in the rumen prior to feeding in both FAU and DEF sheep (24 h since previous meal was offered), although DEF sheep tended to have a higher pre-feeding NO_3 concentration (p = 0.06) (Figure 4.1). Contrastingly, ruminal NO_3 concentration tended to be greater 1.5 h after feeding in FAU relative to DEF sheep (p = 0.06). Nitrate concentration in all sheep, soon after feeding (1.5 h), was much less than may have been expected for sheep consuming 0.44 moles of NO_3. Assuming a rumen volume of 4 L/sheep the expected initial NO_3 concentration was 110 mM. As illustrated in Figure 4.1, the greatest NO_3 concentration 1.5 h after feeding was less than 6 mM, which is less than 5% of possible ruminal NO_3 from dietary NO_3. Similar low concentrations of ruminal NO_3 have also been reported in previous experiments using similar doses of dietary NO_3 (de Raphélis-Soissan et al., 2014; Wang et al., 2018). There are several possible explanations for this result. Firstly, the NO_3 sprayed on the chaff may not have fully dissolved into the rumen fluid. Secondly, NO_3 may have been metabolised within the biofilm (Leng, 2014) of the feed on which it had been sprayed and not released into the whole rumen fluid. A third explanation might be that much of the NO_3 had been rapidly metabolised to NO_2 or NH_3 within 1.5 h after feeding, although this was not supported by the 15 min *in vitro* experiments (Tables 4.2 to 4.5).

Table 4.4 Effect of rumen protozoa (P) on nitrate (NO_3) disappearance rate and nitrite (NO_2) and ammonia (NH_3) accumulation rates *in vitro* in 2.25 mL of liquid (LIQ) or solid (SOL) rumen digesta fractions (RDF) from faunated (FAU) or defaunated (DEF) sheep, incubated for 15 min with 50 mM phosphate buffer containing 1 mM hydrazine sulfate and 30 µg/mL chloramphenicol with the absence (CON) or addition (NO_3) of 5 mM NO_3. Analysed initial concentrations of NO_3, NO_2 and NH_3 in rumen digesta at 0 min are also shown.

	DEF				FAU							
	CON		NO_3		CON		NO_3			p-value		
	LIQ	SOL	LIQ	SOL	LIQ	SOL	LIQ	SOL	SEM	P	NO_3	RDF
Initial concentrations, µmol/L												
NO_3	5	5	459	405	4	5	473	443	30.4	0.35	<0.01	0.13
NO_2	43	44	122	220	59	50	159	220	18.4	0.75	0.02	0.38
NH_3	8289	8511	7614	6938	7914	8267	7026	7175	340.6	0.74	0.14	0.99
Rates, µmol/L/min												
NO_3 disappearance†	0.03^{a}	0.19^{a}	12.12^{b}	19.8^{c}	0.07^{a}	0.12^{a}	9.18^{b}	20.8^{c}	1.774	0.82	<0.01	0.03
NO_2 accumulation‡	0.94^{ab}	2.08^{ab}	31.58^{cd}	49.22^{d}	0.18^{ab}	1.60^{ab}	7.11^{bc}	15.97^{c}	4.092	0.02	<0.01	0.30
NH_3 accumulation	7.45	29.99	20.14	29.89	17.15	14.01	50.31	16.23	5.401	0.80	0.25	0.90

†Interaction NO_3 x RDF p = 0.03; ‡Interaction P x NO_3 p = 0.03

^{a,b,c,d}Means values within a row with different superscript letters denote significant differences between RDF ($p < 0.05$).

Ruminal NO_2 concentration 1.5 h after feeding in FAU tended to be greater ($p = 0.10$) than in DEF sheep; although NO_2 concentrations in DEF tended to be increased 3 h after feeding ($p = 0.06$) (Figure 4.1C - 4.1D) relative to FAU sheep. This pattern suggests that ruminal reduction of NO_3 was slower in the absence of protozoa. With the high between-animal variation in ruminal NO_3 and NO_2 concentrations it was not appropriate to develop an averaged time course of their concentrations in the rumen fluid. However, from the raw values of individual sheep (Figure 4.1) it is apparent that ruminal NO_3 and NO_2 in DEF sheep were consistently much slower to peak than in FAU sheep. These results indicate that the presence of protozoa in the rumen may stimulate the reduction of NO_3 and are in agreement with previous findings (Yoshida et al., 1982; Nakamura & Yoshida, 1991). Protozoa possess a cytoplasmic organelle, the hydrogenosome, that forms hydrogen and contains electron transport carriers which might transfer electrons during NO_3 reduction (Embley et al., 2003; Latham, Anderson, Pinchak & Nisbet, 2016). Further, the concentrations of NO_3 and NO_2 in the rumen fluid were very similar at any point in time, and this was true within FAU and within DEF sheep.

Table 4.5. Effect of rumen protozoa (P) on *in vitro* nitrite (NO_2) disappearance and ammonia (NH_3) accumulation rates in 2.25 mL of liquid (LIQ) or solid (SOL) rumen digesta fractions (RDF) from faunated (FAU) or defaunated (DEF) sheep, incubated for 15 min with 50 mM phosphate buffer containing 1 mM hydrazine sulfate and 30 µg/mL chloramphenicol with the absence (CON) or addition (NO_2) of 5 mM NO_2. Analysed initial concentrations of NO_3 NO_2 and NH_3 in rumen digesta at 0 min are also shown.

	DEF				FAU							
	CON		NO_2		CON		NO_2			*p*-value		
	LIQ	SOL	LIQ	SOL	LIQ	SOL	LIQ	SOL	SEM	P	NO_2	RDF
Initial concentrations, µmol/L												
NO_2	43	44	113	120	59	50	117	113	7.5	0.85	<0.01	0.73
NH_3	8289	8511	7422	7607	7914	8267	7059	7210	184.1	0.34	0.01	0.54
Rates, µmol/L/min												
NO_2 disappearance†	0.94^{ab}	2.08^{a}	0.67^{ab}	0.28^{b}	0.18^{ab}	1.60^{a}	0.50^{ab}	0.33^{b}	0.189	0.27	0.02	0.11
NH_3 accumulation	7.45	29.99	42.32	41.48	17.15	14.01	58.03	46.9	5.020	0.66	<0.01	0.82

†Interaction RDF x NO_2 $p = 0.02$

^{a,b,c,d} Means values within a row with different superscript letters denote significant differences between RDF ($p < 0.05$).

4.4.2. Blood and plasma metabolites

As for ruminal concentrations, substantial between-animal variation was also observed in plasma NO_3 and NO_2 concentrations among both FAU and DEF sheep (Figure 4.2). Plasma NO_3 in FAU sheep ranged between 80 and 1232 µM (CV = 48%) whereas in DEF sheep, it ranged between 55 and 662 µM (CV = 28%). Regarding plasma NO_2, concentrations in FAU sheep ranged between 1 and 11 µM (CV = 35%) while in DEF sheep the range was between 0.5 to 10 µM (CV = 26%). Overall, plasma NO_3 concentrations (Figure 4.2A) did not differ between FAU or DEF sheep throughout the sampling time (p = 0.29). Nitrate and NO_2 concentrations in plasma had not returned to pre-feeding levels after 6 h ($p < 0.06$) (Figure 4.2 A-B) in FAU or DEF sheep, suggesting incomplete reduction of NO_3 to NH_3 and microbial nitrogen synthesis in the rumen. Plasma NO_2 concentrations in FAU sheep (Figure 4.2B) were greater 1.5 h post-feeding (p = 0.04) compared to DEF sheep, suggesting that NO_2 might have been absorbed from the rumen into the bloodstream faster in FAU sheep (Wang, Garcia-Rivera & Burris, 1961). Nitrate and NO_2 may have been excreted in urine and faeces (Lee, Araujo, Koenig & Beauchemin, 2017), metabolised into nitric oxide (Wang et al., 2018), di-nitrogen gas or nitrous oxide (de Raphélis-Soissan et al., 2017) or recycled via saliva and re-processed by gut microbes (Leng, 2008; Nolan et al., 2016).

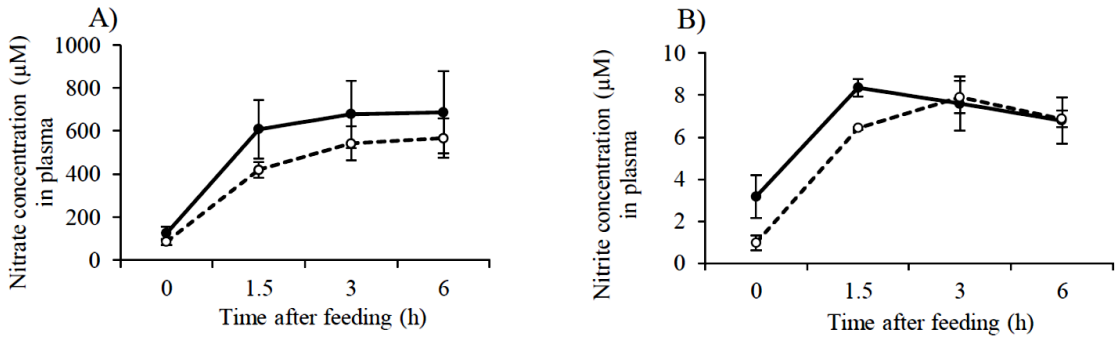

Figure 4.2. Means of nitrate (A) and nitrite (B) concentrations (µM) in plasma of faunated (●; solid lines) or defaunated (○; dashed lines) sheep before (0 h) and after feeding (1.5, 3 and 6 h) a diet containing 1.8% NO3 in DM. Vertical bars indicate standard errors of means (sem). Figure B: note that sem at 1.5 h post feeding are negligible for defaunated sheep.

On average, blood MetHb concentration during the 15 d adaptation period was 1.92 ± 0.26% of total blood haemoglobin and no differences were observed between FAU and DEF sheep (p = 0.18). Time profiles of blood MetHb concentrations in the current research (Figure

4.3) agree with previous findings (Takahashi & Young, 1991; Sar et al., 2004; de Raphélis-Soissan et al., 2014) consistent with NO_2 converting blood haemoglobin to MetHb after feeding of NO_3. Blood MetHb concentration tended to be greater in FAU than in DEF sheep 1.5 h after feeding ($p = 0.08$) but no differences were observed 3 or 6 h post feeding (Figure 4.3; $p > 0.05$). Blood MetHb concentrations peaked in FAU sheep between 1.5 h and 3 h after feeding whereas in DEF sheep MetHb peaked 3 h post-feeding, further suggesting the absence of protozoa from the rumen delays NO_3 reduction. Blood MetHb concentrations at 6 h post-feeding tended to remain above pre-feeding concentrations in both FAU and DEF sheep ($p = 0.06$). Plasma NO_2 concentrations were within the range reported in the literature from similar doses of dietary NO_3 and did not expose animals to NO_2 toxicity (de Raphélis-Soissan et al., 2014). However, in contrast to previous observations (Nakamura & Yoshida, 1991; Nguyen et al., 2016) there were no differences in blood MetHb concentrations between FAU and DEF sheep so there was no evidence of a greater susceptibility to NO_2 toxicity in DEF sheep in this experiment. This discrepancy with Nguyen et al. (2016) could be attributed to differences in the duration of NO_3 supplementation since it has been reported that blood MetHb concentrations increase progressively over time when dietary NO_3 was fed to cattle (Godwin et al., 2015). While Nguyen et al. (2016) found higher blood MetHb concentrations in DEF sheep after 85 days of feeding NO_3, the current experiment was conducted for only 37 days which may have not been long enough to enable differences to develop.

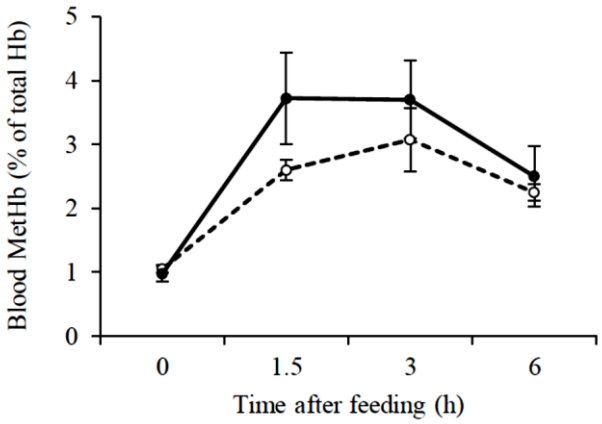

Figure 4.3. Methaemoglobin (MetHb) concentration as percentage of total haemoglobin (Hb) in blood of faunated (-●-, solid lines) or defaunated (-○-, dashed lines) sheep before and after feeding nitrate. Vertical bars indicate standard errors of means.

4.4.3. Protozoa effects on *in vitro* NO_3 reduction in whole rumen digesta, liquid and solid fractions of rumen digesta from faunated and defaunated sheep

Nitrate and NO_2 disappearance rates observed in this experiment were within the range reported in previous findings (Iwamoto et al., 1999). There was no effect of rumen protozoa on NO_3 and NO_2 *in vitro* reduction rates ($p > 0.05$; Tables 4.2 and 4.3). Adding NO_3 to FAU or DEF WRD increased NO_3 disappearance rate relative to the CON digesta ($p < 0.01$; Table 4.2), suggesting that NO_3 reduction is accelerated in response to NO_3 addition (Alaboudi & Jones, 1985; Iwamoto et al., 2001; Shi, Meng, Hou, Ren & Zhou, 2012). Although not significant ($p = 0.21$; Table 4.2), NO_2 accumulation rate was about 2.5 greater in DEF relative to FAU digesta, suggesting the absence of protozoa may increase the risk of NO_2 poisoning in ruminants. Similar findings were observed by Lin et al. (2011) who reported NO_2 accumulation *in vitro* after removal of protozoa from the rumen fluid.

To better understand the direct effect of protozoa on NO_3 metabolism, FAU and DEF rumen digesta was centrifuged to precipitate the protozoa into the SOL fraction and compare the reduction rate relative to bacteria in the LIQ fraction in the second *in vitro* experiment (Tables 4.4 and 5). The interaction between NO_3 treatment and rumen digesta fraction (RDF) was significant ($p = 0.03$). Adding NO_3 increased NO_3 disappearance rate ($p < 0.01$) and this was greater in the SOL relative to the LIQ fraction in both FAU and DEF digesta ($p < 0.05$).

These results confirm that NO_3 reduction in rumen digesta is stimulated by incoming NO_3 as it was observed for *in vitro* for WRD in the current experiment. Regarding NO_2 accumulation rate, the interaction between protozoa status and treatment was significant ($p = 0.03$; Table 4.4). Nitrite was accumulated at a greater rate when NO_3 was added to the SOL fraction of DEF digesta relative to both fractionsof FAU digesta ($p < 0.05$). These *in vitro* results suggest that incoming NO_3 slows NO_2 reduction to NH_3 in the absence of protozoa (Yoshida et al., 1982; Lin et al., 2011).

4.4.4. Effect of protozoa on NO_2 reduction in whole rumen digesta, liquid and solid fractions of digesta from faunated and defaunated sheep

The presence of protozoa in WRD did not affect NH_3 accumulation rate ($p = 0.28$; Table 4.2). Adding NO_3 tended to stimulate NH_3 accumulation relative to CON ($p = 0.07$; Table 4.2). This result may be explained by the fact that both NO_3 and NO_2 reductase activity increases in response to NO_3 exposure in rumen digesta from adapted sheep (Alaboudi & Jones, 1985; Shi et al., 2012) although this effect has not always been reported in the literature (de Raphélis-Soissan, Nolan, Newbold, Godwin & Hegarty, 2016). The addition of NO_2 to the WRD tended to increase NO_2 disappearance rate relative to CON ($p = 0.06$; Table 4.3) although no significant differences were observed in NH_3 accumulation rate ($p > 0.05$).

During the second *in vitro* experiment, the interaction between treatment (NO_2) and RDF was significant ($p = 0.02$; Table 4.5). Adding NO_2 to the SOL fraction of rumen digesta tended to reduce NO_2 disappearance rate in both FAU and DEF ($p = 0.02$) relative to the CON digesta. It seems possible that this result is due to a toxic effect of NO_2 on rumen microorganisms although final NO_2 concentration in the buffer added to the rumen digesta was 0.5 mM which is below the 2 mM NO_2, value reported to inhibit microbial growth (Iwamoto, Asanuma & Hino, 2002). More recently, Roman-Garcia et al. (2019) found that *in vitro* motility and chemotaxis within protozoa were affected differently by NO_2 but not NO_3 for isotrichids versus entodiniomorphids. However, the addition of NO_2 increased NH_3 accumulation rate in both fractions of rumen digesta relative to the CON treatment ($p < 0.01$; Table 4.5), suggesting no adverse effects of NO_2 on rumen microorganisms this current experiment.

As observed for ruminal NO_3 concentrations *in vivo*, recoveries of NO_2 in WRD, LIQ or SOL fractions of rumen digesta *in vitro* were less than expected (Tables 4.3 and 4.5). Low starting NO_2 concentrations may explain the low NO_2 disappearance rate and the lack of

differences in NH_3 accumulation rate between FAU and DEF rumen digesta. This discrepancy in the concentrations of NO_3 and NO_2 could be attributed to NO_3 being stored by rumen bacteria or NO_2, and possibly NO_3, temporally being sequestered in microorganisms in the rumen and/or reversibly bound to either dietary or microbial protein in the rumen, due to NO_2 being a highly reactive molecule (Woolford, Cassens, Greaser & Sebranek, 1976; Leng, 2008). This theory was based on previous research on sheep and cattle fed or dosed with NO_3 and with a rapid disappearance of NO_3 from the rumen and with ruminal NO_3 and NO_2 concentrations below the expected values (Lewis, 1951; Alaboudi & Jones, 1985; Takahashi & Young, 1991; Takahashi, Ikeda, Matsuoka & Fujita, 1998; Sar et al., 2004). However, research on NO_3 storage and NO_2 sequestration by rumen microorganisms in the rumen will need to be undertaken.

4.5. Conclusions

The results from the *in vivo* and *in vitro* experiments indicated that the rate of NO_3 reduction to NH_3 was accelerated by the presence of protozoa and incoming NO_3. The rate of NO_2 accumulation *in vitro* was increased when protozoa were absent and by exposing rumen digesta to NO_3. This results confirms that dietary NO_3 and consequently NO_2, are more rapidly metabolized in the presence of protozoa. Even though no signs of NO_2 poisoning were observed in DEF sheep in this research, defaunation caused greater NO_2 accumulation in the rumen *in vivo* and *in vitro*, increasing the risk of absorption of NO_2 into the blood and possible NO_2 toxicity.

Conflicts of interest

The authors declare no conflicts of interest.

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Dietary nitrate and presence of protozoa increase nitrate and nitrite reduction in the rumen of sheep

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We, the Research 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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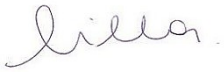

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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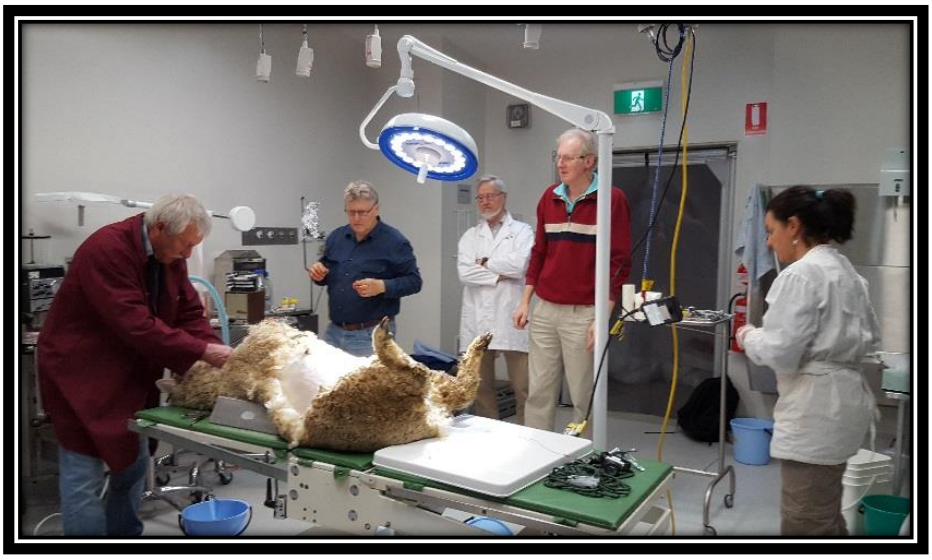
Chapter 5. Nitrate and nitrite absorption, partitioning and excretion by sheep



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Nitrate and nitrite absorption, partitioning and excretion by sheep

5.1. Abstract

A sequence of three experiments was conducted to investigate the absorption, metabolism and excretion of nitrate (NO_3) and nitrite (NO_2) in the gastrointestinal tract (GIT) of sheep, and to determine the fate of NO_3 and NO_2 after intravenous dose of $K^{15}NO_3$ and $Na^{15}NO_2$ in body fluids, tissues and faeces. In Experiment 1, twelve female Merino sheep were assigned to one of two dietary treatments and adapted to the experimental diet over two weeks. Six sheep were fed a control diet of wheaten chaff mixture (600 g wheaten chaff plus 200 g wheat grain, CON) and six sheep were fed the CON diet with the inclusion of 1.8% nitrate/kg DM. After acclimation to the diet, all sheep received an intravenous single dose of $K^{15}NO_3$ and were placed in metabolic cages for daily collection of total faeces and urine over 6 days. Experiment 2 studied movement of an intravenous dose of ^{15}N in body fluids and tissues. Two sheep not adapted to dietary NO_3 were dosed intravenously with ^{15}N-NO_3 or ^{15}N-NO_2 and body fluids and tissue samples were collected one hour after dosing. Finally, Experiment 3 was conducted to identify and quantify the major sites of NO_3 and NO_2 flux within the body, focusing on absorption, partitioning and secretion into the GIT of anaesthetised sheep. A single dose of NO_3 or NO_2 was introduced into the rumen or abomasum or small intestine, and changes in NO_3 and NO_2 concentrations in other pools, including plasma, urine and saliva, were determined. Results from Experiment 1 showed that urinary recovery of ^{15}N-urea 46 h after dosing and total urinary ^{15}N recovery 141 h after dosing were greater in sheep fed the NO_3 diet relative to CON ($P < 0.05$). Recoveries of ^{15}N in tissues indicated that NO_3 and NO_2 were mainly accumulated in the muscle and skin of sheep (Experiment 2). Finally, Experiment 3 indicated that NO_3 and NO_2 were rapidly absorbed from the rumen, abomasum and small intestine into the bloodstream. Nitrite was oxidized in blood plasma and the resultant NO_3 was concentrated and recycled into saliva. Appearance of ^{15}N-urea in urine confirmed the passage of plasma NO_3 to the digestive tract, via saliva or transruminal flow to be reduced by rumen biota to ammonia.

Key words: ^{15}N-urea, rumen, recycling, saliva

5.2. Introduction

The absorption of dietary nitrate (NO_3) through the rumen wall into the bloodstream has been presumed to occur in ruminants as blood MetHb concentration rapidly increased after NO_3 was administered into the rumen of sheep (Lewis, 1951; Wang, Garcia-Rivera & Burris, 1961). However, evidence of direct absorption is limited in the literature (Pfander, Garner, Ellis & Muhrer, 1957). In the study of Lewicki, Garwacki and Wiechetek (1994), nitrite (NO_2) disappearance from the rumen was followed by an increase in plasma NO_3 concentrations, indicating that NO_2 was oxidized to NO_3 in the blood. However, the mechanisms of NO_3 and NO_2 absorption from the rumen into the bloodstream are not entirely understood (Stumpff, 2011).

Nitrate recycling via saliva into the gastrointestinal tract (GIT) in ruminants has been assumed, since previous experiments using ^{15}N-NO_3 reported that ^{15}N appeared in urinary N-urea within 15 min of intravenous injection and continued to increase up to 6 h post dosing (Lewicki, Wiechetek, Souffrant, Karlik & Garwacki, 1998). In the same experiment, appearance of blood ^{15}N-NO_3 as ^{15}N-urea in urine indicated that NO_3 was reduced to ammonia (NH_3) by rumen microorganisms, absorbed, converted to urea in the liver and excreted in urine as urea. Only 38.5% of the administered ^{15}N-NO_3 dose was recovered in urine, suggesting that the remaining NO_3 was metabolized or stored in tissues of sheep (Lewicki et al., 1998). Nitrate reduction by mammalian enzymes has been described in human and in rodent tissues (Jansson et al., 2008). A significant uptake of NO_2 in the liver and kidneys of pigs has also been reported by Eriksson, Yang, Carlström and Weitzberg (2018). Recent findings have shown that the skeletal muscle in rodents serves as a body reservoir for NO_3 which is available for systemic use during dietary NO_3 deprivation; whereas NO_2 is more homogeneously distributed among internal organs, blood and skeletal muscle (Piknova et al., 2015; Gilliard et al., 2018). The objectives of the present series of experiments were to determine the disposal of NO_3 and NO_2 after intravenous dose of ^{15}N-NO_3 and ^{15}N-NO_2, in tissues, urine and faeces (Experiment 1 and 2), and to investigate the absorption, metabolism and excretion of NO_3 and NO_2 in the GIT of sheep (Experiment 2).

5.3. Materials and methods

5.3.1. Experiment 1. Recovery of ^{15}N labelled nitrate in urine and faeces in sheep

5.3.1.1. Animals and dietary treatments

All procedures involving animals were performed in accordance with the University of New England Animal Ethics Committee (AEC approval no. 16/068). Twelve female Merino sheep (27.5 ± 0.77 kg; 12 months of age) were selected according to live-weight (LW) and randomly assigned to one of two dietary treatments using stratified randomization. Sheep were housed in individual pens and acclimated to a basal diet over two weeks prior to the start of the experiment with *ad libitum* access to feed and clean water. The basal diet was a wheaten chaff mixture (600 g wheaten chaff plus 200 g wheat grain, CON). The second dietary treatment consisted of the basal diet plus the inclusion of 1.8% nitrate/kg DM (NO_3, provided as 3.14% calcium nitrate, $5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway). Acclimation to NO_3 was achieved by sequentially increasing the NO_3 diet concentration as follows: NO_3 at 0.9% of DM from Day 1 to Day 7, NO_3 at 1.4% of DM from Day 8 to Day 14 and NO_3 1.8% of DM from Day 15 to d 28. Nitrate treated straw was prepared weekly by spraying dissolved calcium nitrate onto the wheaten chaff while mixing in a ribbon mixer.

5.3.1.2. Marker introduction and sampling procedure

A single dose of ^{15}N-labelled potassium nitrate ($K^{15}NO_3$, 99% ^{15}N, Cambridge Isotope Lab., MA, USA) was administered intravenously as a single injection (22 mg ^{15}N-NO_3) for the purpose of studying ^{15}N-NO_3 excretion in urine and faeces. After marker introduction, sheep were placed in metabolism cages and daily total collection of faeces, urine and feed refusals was conducted for 6 days. The 12 sheep were randomly allocated between 2 consecutive periods (n = 6 animals per period). Animals were offered 800 g (fresh weight) of their experimental diet. The daily ration was offered at 1300 h on Day 1 and then at 0900 h and 1700 h on the subsequent days. Both offered and refused feed were weighed daily. Faeces were collected and weighed at 1700 h, 2000 h, 2400 h on Day 1, at 0600 h, 0900 h, 1200 h and 1800 h on Day 2 and then at 0900 h and 1700 h on Day 3, Day 4, Day 5 and Day 6 until 0900 h on Day 7, comprising a 141 h period.

Urine collection and preservation was conducted according to the procedure of IAEA (1997). A 50 mL subsample of urine was collected at 0900 h and 1700 h during Day 1 and Day 2 of each period for determination of ^{15}N and ^{15}N-urea excreted in urine during 46 h. A 100 mL subsample was collected daily at 0900 h from Day 3 to Day 7 for determination of total ^{15}N and ^{15}N-urea excreted in urine. Urine samples were stored at -20 °C for subsequent analysis. A subsample of 10 mL of the total urine total collected was concentrated by freeze drying and 2 mL of MilliQ® water then added. A 0.5 mL subsample was used for ^{15}N determination and another 0.5 mL was alkalinized using potassium carbonate and incubated with Jack Bean Urease (Sigma, Type IX), according to Conway and O'Malley (1942) and Fawcett and Scott (1960), to determine urea-N contents. Diffusion and collection of ammonia was performed as described by de Raphélis-Soissan et al. (2017). From the homogenised faecal output in each interval, a 50 g fresh fecal subsample was taken, oven dried to constant weight at 65°C and ground though a 1-mm sieve for further analysis. The abundance of ^{15}N in urine and fecal samples was analysed with a Sercon 20-22 continuous flow isotope-ratio mass spectrometer connected to an ANCA-GSL sample preparation unit (Sercon Limited, Gateway, Crewe, Cheshire, UK).

5.3.2. Experiment 2. Distribution of intravenous doses of ^{15}N-labelled nitrate and nitrite in sheep

The main objective of Experiment 2 was to study the disposal of an intravenous dose of $K^{15}NO_3$ or $Na^{15}NO_2$ in body fluids and tissues, 60 min after dosing two five-year-old sheep (45.3 kg LW) non-adapted to dietary NO_3 supplementation. All animal care protocols were approved by the Animal Ethics Committee (AEC16-077) of the University of New England, Armidale, NSW.

5.3.2.1. Surgical procedure preparation

One sheep per day was taken to the surgery and their wool was clipped from the ventral and abdominal regions. Cable ties were applied to rumen cannulas to prevent leakage of ruminal contents. Anaesthesia was induced by intravenous injection of sodium thiopentone (13 mg/kg LW). An endotracheal tube was then passed into the trachea and anaesthesia was maintained with inhalational isoflurane (~2 % in oxygen). A second endotracheal tube was then passed into the oesophagus and inflated to prevent regurgitation of ruminal contents. The front of the surgical table was reclined slightly so the head sloped downward, allowing saliva to drain from

the mouth and be collected as it was excreted from the oral cavity while animals were under anaesthesia. Polyethylene catheters (60 cm of length, OD 1.50 mm x ID 1.00 mm, Sterihealth, VIC, Australia) were inserted into the left-side jugular vein of each sheep through a double bevel introducer needle (16G, Surflo I.V. catheter, Tokyo, Japan). The jugular blood sampling catheter was filled with 100 IU heparinised saline between sample collections. A Foley balloon catheter (10 FG) was inserted into the bladder to facilitate urine collection.

5.3.2.2. Gastrointestinal tract clamping, tracers injection and sampling procedures

Experimental procedures for dosing and sampling are summarized in Table 5.1. An abdominal laparotomy was performed to the left of the linea alba in the epigastric region. In sheep 1 and 2, clamps were placed on the pylorus, reticulo-omasal orifice and the ileo-caecum junction with bowel forceps. Before the injection of tracer was commenced, samples of semitendinosus muscle, skin, blood, saliva, urine, and digesta from rumen, abomasum, small intestine and caecum were taken. Background samples for pancreas, liver, gallbladder contents and spleen were taken from another sheep prior to euthanasia, with all samples used to estimate the background of ^{15}N in the tissue or digesta. Background samples of saliva (10 mL), abomasal fluid (10 mL), gallbladder fluid (5 mL), small intestine (10 mL), caecum fluid (5 mL), and rumen fluid (10 mL) were preserved with 1 mL 38 % formaldehyde. Saliva was collected from the oral cavity while animals were under anaesthesia. Samples were stored at -20 ˚C for later analysis.

Sheep were dosed intravenously with either $K^{15}NO_3$ (Sheep 1; 25.09 mg ^{15}N, 99 atoms %, Cambridge Isotope Lab., MA, USA) or $Na^{15}NO_2$ (Sheep 2; 10.05 mg, CIL, 98+ atoms %, Cambridge Isotope Lab., MA, USA). Two blood samples were taken from a jugular vein via catheter at 10, 20, 30, 40, 50, and 60 min post injection. Catheters were flushed with heparinised saline after each sampling. A 3 mL sample immediately had MetHb percentage determined by blood gas analyser (ABL 800 Flex, Radiometer, Brønshøj, Denmark), while a 10 mL whole blood sample was collected in heparinised vacutainers and frozen at -20 ˚C for later analysis. Total urine and saliva output were recorded and 10 mL subsamples were collected and preserved with 38% formaldehyde. After 60 min, sheep were euthanized with sodium pentobarbital. Samples (10 mL) from abomasum, gallbladder, small intestine and caecum contents were collected and preserved with formaldehyde after the total gravimetric volume of each organ was recorded. Total ruminal content was recorded and 50 mL aliquots were taken;

one preserved with 1 mL concentrated (98%) H_2SO_4 and the other with 1 mL 38 % formaldehyde. Biopsies to evaluate the appearance of ^{15}N in tissue were taken from skin, muscle (semitendinosus), liver, spleen and pancreas. These tissues were rinsed in saline solution to remove non-adherent bacteria and digesta and stored at -20 ˚C.

5.3.3. Experiment 3. Nitrate and nitrite absorption and excretion in sheep

This experiment sought to identify and quantify the major sites and rates of NO_3 and NO_2 flux within the body of sheep, focusing on absorption, partitioning and excretion. This was done by introducing a single dose of NO_3 or NO_2 into the isolated rumen or abomasum or small intestine of anaesthetised sheep and monitoring changes in NO_3 and NO_2 concentrations in other pools including plasma, urine and saliva.

Experiment 3 was conducted over 21 days. All animal care protocols were approved by the Animal Ethics Committee (AEC16-077) of the University of New England, Armidale, NSW. Six rumen (and in some cases abomasal) cannulated crossbred female sheep (56 ± 8.1 kg) were housed in individual pens and randomly assigned to one of 6 NO_3 or NO_2 dosing protocols (Table 5.1). Animals were acclimated over 14 d to a mixed blend of lucerne and oaten chaff (Manuka Feeds, Quirindi, NSW) with inclusion of 2% nitrate per kg as-fed, provided as 3.14 % calcium nitrate ($5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway). The diet was prepared weekly by spraying dissolved calcium nitrate onto the chaff while mixing in a ribbon mixer. The daily ration was offered once per day at 0830 h. Fresh water was available *ad libitum.* Food was withheld for 24 h and water for 12 h before each individual animal was dosed and studied for animal safety during surgery. Surgical procedure preparation was performed as for Experiment 2.

5.3.3.1. Rumen procedure

Experimental procedures for dosing and sampling are summarized in Table 5.1. An abdominal laparotomy was performed in Sheep 2 to 8 along the right ventral midline in the epigastric region. In sheep 3 the reticular-omasal orifice was hidden in surgical adhesions from a prior procedure, the pyloric region of the abomasum was clamped with bowel forceps. A sample probe with the end enclosed in a dacron bag filter (40 μm pore size) was introduced through a purse string suture in the rumen wall, with the suture tightened to prevent leakage of rumen contents. In Sheep 3, the rumen was dosed with 1038 mg NO_3-N ($NaNO_3$, Sigma-Aldrich ≥ 99

and 7881 mg L-cysteine hydrochloride (Sigma, anhydrous ≥ 98 %), dissolved in 500 mL MilliQ® water. The reticulo-rumen was manually massaged and stirred with the indwelling probe to facilitate mixing of the infusate. Sheep 4 underwent a similar procedure as for the sheep rumen dosed with NO_3-N, excepting that the reticulo-omasal orifice was clamped and the rumen was dosed with 514.5 mg NO_2-N ($NaNO_2$, Sigma-Aldrich ≥ 99 %) and 7883 mg L-cysteine hydrochloride (Sigma, anhydrous ≥ 98 %), dissolved in 500 mL MilliQ® water. Cysteine was included to inhibit microbial reduction of NO_3 (Takahashi, Johchi & Fujita, 1989).

5.3.3.2. Abomasum procedure

The abdomen was opened to the right of the midline in the epigastric region. The abomasum was clamped at the pylorus, and at the anterior end of the abomasum. A sample tube with end enclosed in a porous dacron bag was introduced through a purse string suture in the abomasal wall similar to the rumen procedure. Sheep 5 was dosed with 1065 mg NO_3-N ($NaNO_3$) dissolved in 100 mL MilliQ® water. Sheep 6 was dosed with 212 mg ($NaNO_2$) dissolved in 100 mL MilliQ® water. In this last case, the dose of NO_2-N received was less than intended since the abomasum in this animal was atrophied and the smaller volume was insufficient to receive the whole dose.

5.3.3.3. Small intestine procedure

An abdominal laparotomy was performed to the left of the linea alba in the epigastric region. A length (162 cm) of small intestine was isolated with ligatures and a PVC catheter (3 mm OD) was inserted via a small incision into the proximal end of the intestinal loop, and passed until close to the distal end. The test infusate was then introduced as the catheter was slowly drawn proximally to ensure an even distribution of dose. Sheep 7 was dosed with 1062 mg NO_3-N ($NaNO_3$) dissolved in 100 mL MilliQ® water, while Sheep 8 was dosed with 515 mg ($NaNO_2$) dissolved in 100 mL MilliQ® water. The catheter was then removed and the incision closed. Intestinal samples were taken by puncturing the intestinal loop with a needle and withdrawing contents by syringe.

Table 5.1. Experimental procedures for dosing, sampling and chemical determinations

	Experiment 1	Experiment 2		Experiment 3					
Sheep	n = 12	1	2	3	4	5	6	7	8
Dose compound	$K^{15}NO_3$	$K^{15}NO_3$	$Na^{15}NO_2$	NO_3	NO_2	NO_3	NO_2	NO_3	NO_2
Dose (mg N)	22	25	10	1038	515	1065	212	1062	515
L-cysteine (mg)				7881	7883				
Dose site	Jugular vein	Jugular vein		Rumen		Abomasum		Small intestine	
Clamped or ligature site		Pylorus, reticulo-omasal orifice and ileo-caecum junction.		Pylorus and reticulo-omasal orifice.		Pylorus and anterior end of abomasum.		Ligatures to isolate 162 cm section.	
Sample	Urine and faecal samples	Urine, saliva, blood, skin, muscle, liver, faeces, spleen. Contents from rumen, abomasum, small intestine, caecum, gallbladder		Urine, saliva and blood. Contents from rumen		Urine, saliva and blood. Contents from abomasum		Urine, saliva and blood. Contents from small intestine	
Sample time	Total collection of urine and faeces over 6 days	Before dosing (-10 and 0 min) and 60 min after dosing		Before dosing (-10 and 0 min) and 10, 20, 40, 60, 80, 100 and 120 min after dosing					
Chemical analysis	Enrichment of ^{15}N in urine and faeces	Blood MetHb concentration. Enrichment of ^{15}N in GIT contents, urine, saliva and blood.		Blood MetHb concentration. Concentration NO_3 and NO_2 in GIT contents, urine, saliva and blood.					

5.3.4. Sampling procedure

Background samples (-10, 0 min) of blood, saliva, urine and rumen, abomasum or duodenum contents were collected once anaesthesia was established, but before any NO_3 or NO_2 dose was introduced. Two blood samples were taken from a jugular vein via catheter at 10, 20, 40, 60, 80, 100, 120, 140, 160 and 180 mins after dosing. Blood methaemoglobin (MetHb) as percentage of total haemoglobin was immediately determined in a blood gas analyser (ABL 800 Flex, Radiometer, Brønshøj, Denmark) while 10 mL blood samples were collected in heparinised vacutainers and held on ice until centrifugation for 15 mins at 1050*g* at 4˚C (Beckman Coulter TJ-6, USA) for plasma separation. Plasma samples (5 mL) were diluted 1:1 with 95 % ethanol and stored at -20°C until subsequent analysis. Samples of saliva and urine, and rumen, abomasum or duodenal contents were collected at 10, 20, 40, 60, 80, 100, 120, 140, 160 and 180 min after dosing. Subsamples (5 mL) were preserved with 0.8 mL 38% formaldehyde for subsequent NO_3 and NO_2 determinations. Urine volume excreted in each interval was recorded. A second 5 mL rumen fluid sample was preserved with 3 drops of concentrated sulfuric acid (H_2SO_4; 98%) for subsequent determination of rumen NH_3-N concentration. All samples were stored at -20˚C until further analysis. Each experimental sheep was euthanized with sodium pentobarbital following their surgical procedure and sampling.

5.3.5. Sample analysis

Rumen, abomasum and duodenum content pH was determined at the time of sample collection with a portable pH meter (Orion 230 Aplus, Thermo Scientific, USA). Determination of NO_3 and NO_2 in plasma, saliva, urine, abomasum and duodenum samples were performed by a continuous flow analyser (SAN ++, Skalar, Breda, Netherlands), as described by de Raphélis-Soissan et al. (2017). From sheep dosed into the rumen with NO_3 or NO_2 with added L-cysteine, rumen fluid, plasma, saliva and urine samples were incubated with denitrifying bacteria lacking nitrous oxide (N_2O) reductase activity (Sigman et al., 2001). This was done as a precursor to (NO_3+NO_2) analysis by mass spectrometry as cysteine was found to interfere with colorimetric determination of NO_3 by the Griess reagent (Bellavia, Kim-Shapiro & King, 2015). Nitrous oxide subsequently produced, after complete bacterial reduction of NO_3 and NO_2 present in the samples, was quantified as N_2 on a Thermo Trace GC Ultra (Thermo Fisher, Waltham, MA, USA) interfaced to a Thermo Delta V Plus (Thermo Fisher Scientific, Bremen, Germany) isotope-ratio mass spectrometer.

This mass spectrometer was only used to determine ion concentration not isotope ratios. Because of possible effect of NO_2 on microbes, mass spectrometry data was pooled for (NO_3+NO_2). A Dade Behring Dimension Xpand Plus Integrated Chemistry Analyser (Ramsey, Minnesota, USA) was used to determine blood urea N in plasma.

Frozen tissues, whole blood, plasma, saliva and digesta samples were subsequently freeze-dried and ground using glass balls and a vortex. Total N concentration was determined by a simultaneous carbon and nitrogen elemental analyser (Leco Corporation TruSpect, St Joseph. MI). The abundance of ^{15}N in Experiments 1 and 2 was analysed with a Sercon 20-22 continuous flow isotope-ratio mass spectrometer connected to an ANCA-GSL sample preparation unit (Sercon Limited, Gateway, Crewe, Cheshire, UK).

5.4. Results

5.4.1. Experiment 1: Excretion of urinary urea, nitrate and recovery of ^{15}N-labeled nitrate in urine and faeces

No significant difference between sheep fed NO_3 or CON diets was observed in the proportion of the intravenous ^{15}N-NO_3 dose recovered in urine as total ^{15}N after 46 h (P = 0.22; Table 5.2). However, average recovery of the ^{15}N-NO_3 dose as ^{15}N-urea after 46 h was 52% greater in sheep supplemented with NO_3 than CON (P = 0.04). After 141 h, recovery of ^{15}N as percentage os the total $^{15}NO_3$ was significantly greater in sheep fed the NO_3 diet (P = 0.002) but no treatment differences were observed in the recovery of ^{15}N in urea after 141 h (P = 0.85).

Table 5.2. Recovery of ^{15}N (as % of total ^{15}N dose) in urine or faeces at 46 h or 141 h after intravenous injection of ^{15}N-NO_3 in sheep fed a control diet of wheaten chaff plus wheat grain (CON) or supplemented with 18 g NO_3/ kg DM (NO_3).

	CON	NO_3	SEM	P-value
Total ^{15}N recovered in urine after 46 h (%)	23.4	31.3	3.39	0.18
Total ^{15}N recovered in urine after 141 h (%)	33.4	48.8	3.32	<0.01
^{15}N removed in urine urea after 46 h (%)	14.0	27.0	3.55	0.04
^{15}N removed in urine urea after 141 h (%)	17.2	19.8	4.2	0.85
Total ^{15}N recovered in faeces after 141 h (%)	17.2	14.1	1.27	0.26

Total fecal recovery of the intravenously injected ^{15}N-NO_3 after 141 h accounted for 15.6% of the dose (average 14.1-17.2%) and did not differ between dietary treatments (P = 0.26; Table 5.2). Over the 141-h faecal collection, the recovery of ^{15}N increased throughout the experimental period but had largely plateaued by 100 h (Figure 5.1).

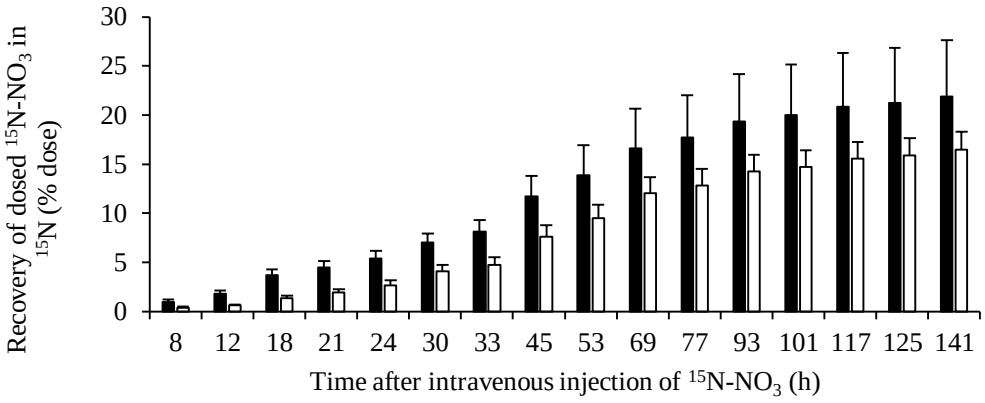

Figure 5.1. Recovery of intravenously administered ^{15}N-NO_3 as ^{15}N in faeces (% of injected ^{15}N-NO_3) in sheep fed a control diet of wheaten chaff plus wheat grain (CON; □) or supplemented with 18 g nitrate/ kg DM (NO_3; ■). Vertical bars indicate SEM.

5.4.2. Experiment 2: Short term fate of ^{15}N in sheep tissues after intravenous ^{15}N-NO_3 or ^{15}N-NO_2 administration.

Average blood MetHb concentrations were below 2% in both sheep dosed with $^{15}NO_3$ (1.06±0.03) or $Na^{15}NO_2$ (1.58±0.17). The highest recoveries of ^{15}N from ^{15}N-NO_3 and ^{15}N-NO_2 in sheep dosed intravenously and euthanized 60 min later, were observed in skin,

abomasum, muscle, saliva and liver (Table 5.3). Less than 1% of the intravenous dose of $^{15}NO_3$ or $^{15}NO_2$ was recovered in caecum content, urine, pancreas, gallbladder content and faeces. Recovery of ^{15}N in the small intestine was greater in sheep dosed with $^{15}NO_2$. No enrichment was observed in the rumen, spleen or faeces of sheep dosed with either $^{15}NO_3$ or $^{15}NO_2$.

Table 5.3. Recovery of intravenously injected ^{15}N-NO_3 and ^{15}N-NO_2 (% of dose) in tissues and body fluids 60 min after dosing sheep with 25.1 mg ^{15}N-NO_3 or 10.05 mg ^{15}N-NO_2.

Sample	^{15}N-NO_3 (%)	^{15}N-NO_2 (%)
Skin	22.8	26.9
Abomasum	6.8	3
Whole Blood	22.1	20.3
Plasma	6.7	8.5
Muscle	4.5	12.9
Saliva	3.9	2.5
Liver	1.4	1.4
Small Intestine	0.7	1.5
Caecum	0.5	0.4
Urine	0.4	0.6
Pancreas	0.1	0.2
Gallbladder	0	0.2
Spleen	0	0
Faeces	0	0
Rumen	0	0
TOTAL	69.9	78.5

5.4.3. Experiment 3

5.4.3.1. Nitrate absorption from the rumen, abomasum and small intestine of sheep.

Dosing sheep with $NaNO_3$ into the rumen resulted in a rapid absorption of (NO_3+NO_2) from the rumen and appearance into blood plasma (Table 5.4). Concentrations of (NO_3+NO_2) in saliva and urine started to increase 40 min after dosing with raised concentrations persisiting at leats up to 160 min (Table 5.4).

Table 5.4. Progressive appearance of (NO_3+NO_2) (mmol N/L) in rumen content, plasma, saliva and urine, after dosing with 1000 mg NO_3-N into the isolated rumen of an anaesthetised sheep.

Time (min)	Rumen contents	Plasma	Saliva	Urine
	NO_3+NO_2	NO_3+NO_2	NO_3+NO_2	NO_3+NO_2
0	0.4	0.0	0.3	0.2
10	31.4*	0.3	0.0	1.4
20	12.7	0.8	1.0	2.1
40	13.0	0.6	3.2	5.6
60	14.2	1.2	6.4	9.1
80	9.5	1.1	na	na
100	10.1	0.8	8.2	8.8
120	8.6	na	8.6	9.5
140	7.3	1.4	9.1	7.6
160	3.3	0.9	9.3	7.9
180	1.2	na	na	na

na: data not available

*concentration exceed that expected (dose/rumen volume) so may reflect poor initial mixing of dose in digesta

Following the introduction of the dose of unlabelled $NaNO_3$ into the abomasum, NO_3 was rapidly absorbed raising plasma NO_3 concentrations, but not plasma NO_2, immediately after dosing (Table 5.5). Urinary and salivary NO_3 started to increase within 20 min of dosing, with urinary NO_3 concentration peaking 80 min post-dosing, and salivary NO_3 still rising 180 min post-dosing (Table 5.5).

Table 5.5. Progressive appearance of NO_3 (mmol N/L) and NO_2 (mmol N/L) in abomasal content, plasma, saliva and urine, after dosing with 1000 mg NO_3-N into the isolated abomasum of an anaesthetised sheep.

	Abomasum content		Plasma		Saliva		Urine	
Time (min)	NO_3	NO_2	NO_3	NO_2	NO_3	NO_2	NO_3	NO_2
-10	0.1	0.1	0	0	na	na	0.2	0
0	0.1	0.2	0	0	0.1	0	0.2	0
10	578.4	1.1	0.1	0	0.1	0	0.1	0
20	707.4	1	0.3	0	0.1	0	1.6	0
40	419.9	1.1	0.4	0	0.6	0.1	4.7	0
60	345.9	1	0.5	0	1.5	0.1	6.7	0
80	342.3	1	0.6	0	2	0.2	7.0	0
100	307.2	1	0.7	0	2.7	0.2	6.2	0
120	254.8	1	0.7	0	2.2	0.2	5.4	0
140	211.9	1	0.7	0	3.7	0.2	4.5	0
160	177.3	1.1	0.7	0	3.7	0.2	2.9	0
180	128.1	1.1	0.7	0	4	0.2	1.4	0

na: data not available

Nitrate was also rapidly absorbed from the small intestine after $NaNO_3$ injection (Table 5.6). There was a 22-fold increase in the concentration of NO_3 in saliva after 180 min relative to NO_3 concentration 10 min after dosing. In contrast, NO_3 concentration in plasma and urine only slightly increased over 180 min. Nitrite concentration in the small intestine after dosing sheep with NO_3 was insignificant. Only negligible concentrations of NO_2 were detected in saliva, and no NO_2 was detected in urine or plasma.

Table 5.6. Progressive appearance of NO_3 (mmol N/L) and NO_2 (mmol N/L) in small intestine content, plasma, saliva and urine, after dosing with 1000 mg NO_3-N into the isolated small intestine of an anaesthetised sheep.

	Small Intestine content		Plasma		Saliva		Urine	
Time (min)	NO_3	NO_2	NO_3	NO_2	NO_3	NO_2	NO_3	NO_2
-10	0.1	0.0	0.0	0.0	0.7	0.0	1.0	0.0
0	0.4	0.0	0.0	0.0	0.3	0.0	0.7	0.0
10	460.1	2.7	0.7	0.0	0.6	0.1	1.7	0.0
20	279.5	2.6	1.3	0.0	1.2	0.1	0.3	0.0
40	137.1	2.5	2.0	0.0	6.5	0.2	1.5	0.0
60	111.0	2.6	2.5	0.0	12.7	0.3	2.5	0.0
80	100.3	2.5	2.9	0.0	15.7	0.3	2.8	0.0
100	93.6	2.5	2.7	0.0	17.8	0.3	2.2	0.0
120	97.7	2.4	2.6	0.0	13.6	0.3	2.1	0.0
140	110.9	2.6	2.4	0.0	12.6	0.3	2.1	0.0
160	90.9	2.3	2.6	0.0	14.6	0.3	na	na
180	80.3	2.4	2.5	0.0	14.1	0.3	1.8	0.0

na: data not available

5.4.3.2. Nitrite absorption from the rumen, abomasum and small intestine of sheep.

There was a rapid decrease in (NO_3+NO_2) concentrations in the rumen contents after dosing sheep intraruminally with $NaNO_2$ (Table 5.7). Plasma, salivary and urinary concentrations of (NO_3+NO_2) increased inmediately after dosing, but appeared to have plateaued before160 min. Over some sampling intervals there was no urine produced after dosing sheep with $NaNO_2$ in the rumen (Table 5.7).

Table 5.7. Progressive appearance of NO_3+NO_2 (mmol N/L) in rumen content, plasma, saliva and urine, after dosing with 500 mg NO_2-N into the isolated rumen of an anaesthetised sheep.

Time (min)	Rumen content	Plasma	Saliva	Urine
	NO_3+NO_2	NO_3+NO_2	NO_3+NO_2	NO_3+NO_2
0[§]	0.0	0.0	0.1	0.1
10	1.8	0.5	1.7	na
20	3.5	0.8	1.9	0.4
40	1.4	0.6	3.7	0.8
60	0.8	1.1	na	na
80	0.3	1.3	6.1	1.2
100	0.6	2.2	na	1.7
120	0.0	0.9	2.4	no sample
140	0.0	0.7	7.9	na
160	0.1	2.1	na	no sample

na: data not available

As for sheep dosed with NO_3 into the abomasum, NO_2 was apparently lost from the abomasum of sheep dosed with NO_2 (Table 5.8). Concentrations of NO_2 in saliva showed only small rises over time and had not begun to decline 180 min after NO_2 had been introduced into the abomasum. Negligible NO_2 was detected in plasma and urine. Nitrate concentrations in plasma, saliva and urine increased after abomasal NO_2 dosing and the raised concentrations were sustained out to 180 min. Salivary NO_3 increased dramatically, becoming 10-fold greater that concentrations in plasma by 60 min after dosing, and 20-fold greater by 180 min. There was almost no rise in plasma NO_2 concentrations after dosing NO_2 into the abomasum (Table 5.8).

Table 5.8. Progressive appearance of NO_3 (mmol N/L) and NO_2 (mmol N/L) in abomasal content, plasma, saliva and urine, after dosing with 500 mg NO_2-N into the isolated abomasum of an anaesthetised sheep.

	Abomasal content		Plasma		Saliva		Urine	
Time (min)	NO_3	NO_2	NO_3	NO_2	NO_3	NO_2	NO_3	NO_2
-10	na	na	0	0	0.2		0.1	0
0	0.1	0	0	0	0.2	0	0.1	0
10	0	249.7	0.1	0	0.2	0.1	na	na
20	0	195.6	0.1	0	0.4	0.1	na	na
40	0	151.1	0.1	0	0.8	0.1	0.1	0
60	0	129.6	0.1	0	1	0.2	0.1	0
80	0	109.4	0.1	0	1.4	0.2	0.2	0
100	0	102	0.1	0	1.4	0.2	0.8	0
120	0	88.2	0.1	0	1.6	0.2	1	0
140	0	81.2	0.2	0	2.1	0.2	0.8	0
160	0	81.7	0.1	0	2.1	0.2	na	na
180	0	58	0.1	0	2.2	0.2	0.7	0

na: data not available

There was a rapid disappearance of NO_2 from the small intestine after dosing the intestine with NO_2 (Table 5.9). Plasma and salivary NO_2 markedly increased over time. As for sheep dosed with NO_2 in the abomasum, urine production after dosing sheep with NO_2 into the small intestine was not continuous. Nitrate concentration appeared to have plateaued in plasma and saliva by 180 min after dosing, but was still incresaing in urine. Salivary NO_3 concentrations remained on average 5-fold greater than plasma concentrations. Nitrate concentrations in urine reached a 10-fold increase over plasma concentrationes after 120 min post-dosing (Table 5.9).

Table 5.9. Progressive appearance of NO_3 (mmol N/L) and NO_2 (mmol N/L) in small intestine content, plasma, saliva and urine, after dosing with 500 mg NO_2-N into the isolated small intestine of an anaesthetised sheep.

	Small intestine content		Plasma		Saliva		Urine	
Time (min)	NO_3	NO_2	NO_3	NO_2	NO_3	NO_2	NO_3	NO_2
-10	na	na	0.0	0.0	0.1	0.0	0.1	0.0
0	0.1	0.0	0.0	0.0	0.1	0.0	0.1	0.0
10	0.0	229.1	0.1	0.1	0.1	0.0	0.1	0.0
20	0.0	185.5	0.2	0.2	0.3	0.2	na	na
40	0.0	121.6	0.3	0.2	0.4	0.2	0.3	0.1
60	0.0	81.2	0.5	0.3	1.0	0.3	na	na
80	0.0	82.5	0.6	0.2	3.4	0.9	0.4	0.0
100	0.0	37.5	0.8	0.2	3.3	0.9	0.6	0.0
120	0.0	22.0	0.8	0.1	4.5	0.9	7.8	0.2
140	0.0	30.2	0.9	0.1	4.9	0.6	9.8	0.2
160	0.0	5.6	0.9	0.0	6.0	0.5	11.0	0.2
180	0.0	6.6	0.9	0.0	4.4	0.4	10.4	0.1

na: data not available

5.4.3.3. Urine production and urinary recovery of NO_3 and NO_2

Total urine volume after dosing sheep with NO_3 or NO_2 into the rumen, abomasum or small intestine are shown in Table 5.10. Urine production after dosing sheep with NO_3 into the rumen, abomasum or small intestine was continuous, whereas adding NO_2 caused an interruption in urine production immediately after dosing (data not shown).Urinary recovery of (NO_3+NO_2) in sheep dosed with NO_3 was less variable than in sheep dosed with NO_2 (Table 5.10).

Table 5.10. Total urinary recovery of NO_3+NO_2 (as % of the dose) 180 min, after dosing sheep with 1000 mg NO_3-N or 500 mg NO_2-N into the isolated rumen, abomasum or small intestine.

Site / dose	Urine volume (mL)	Urinary recovery of (NO_3+NO_2) as % dose
Rumen + NO_3	124.5	9.1
Rumen + NO_2	53.0	4.0
Abomasum + NO_3	99.5	5.6
Abomasum + NO_2	32.5	1.4
Small Intestine + NO_3	57.5	1.5
Small Intestine + NO_2	84.0	20.3

5.4.3.4. Blood MetHb concentrations

After administration of $NaNO_3$ into the rumen, there was a slightly increase in blood MetHb concentrations (Figure 5.2), with the highest value being 4.2% of total Hb (Figure 5.2A). Blood MetHb concentrations in sheep dosed with $NaNO_3$ into the abomasum and small intestine were less than 2% of total Hb and remained stable throughout the 180 min.

Concentrations of blood MetHb in sheep dosed with $NaNO_2$ into the rumen peaked 20 min after dosing, with the peak value being 19.6% of total Hb (Figure 5.2B).There was almost no rise in blood MetHb concentrations after dosing Sheep 6 with $NaNO_2$ into the abomasum (Figure 5.2B). However, dosing $NaNO_2$ into the small intestine led to high blood MetHb concentrations between 4% and 48% of total Hb (Figure 5.2B).

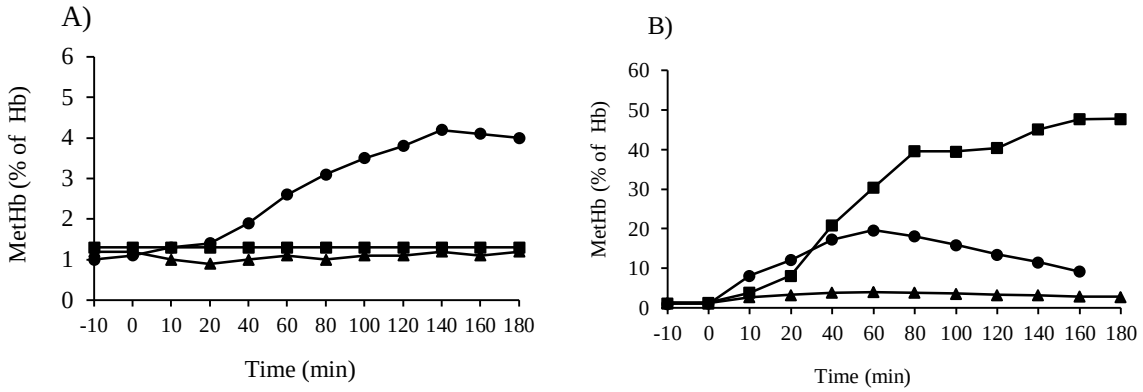

Figure 5.2. Methaemoglobin (MetHb) concentration as percentage of total haemoglobin (Hb) in blood of anaesthetized sheep dosed with 1000 mg of NO_3-N (A) or with 500 mg of NO_2-N (B) into the rumen (-●-), abomasum (-▲-) or small intestine (-■-)

5.5. Discussion

The main objectives of the present experiments were to investigate the absorption, recycling and excretion of NO_3 and NO_2 in the GIT of sheep, and after intravenous dose of labelled $^{15}NO_3$ and $^{15}NO_2$ to determine the fate of NO_3 and NO_2 into tissues, urine and faeces in sheep. The key observations of this work were that NO_3 and NO_2 were rapidly absorbed from the GIT into the bloodstream, concentrated in plasma, then recycled via saliva into the GIT as well as being partially accumulated in several tissues, or excreted in the urine of sheep.

5.5.1. Experiment 1

The results obtained in Experiment 1 showed that almost half of the intravenous dose of ^{15}N-NO_3 was excreted in urine after 141 h in sheep supplemented with NO_3 which was significantly greater than the CON group ($P < 0.01$). Previous studies have reported urinary recoveries of ^{15}N-NO_3 between 16% and 38.5% (Lewicki et al., 1998) after 50 h. The recovery of ^{15}N-NO_3 in urine as urinary ^{15}N-urea in particular, confirms the passage of plasma NO_3 to the digestive tract. This must have occurred as there is no mammalian process to convert NO_3 to urea. Blood $^{15}NO_3$ must have entered the digestive tract via saliva or transruminal flow, and was reduced by rumen biota to NH_3 or amino acids that could be absorbed and subsequently be converted to urea in the liver. The results for ^{15}N and ^{15}N-urea obtained in Experiment 1 indicate that there is recycling of NO_3 into the GIT. This could be expected to improve the N balance in the animals by allowing them to gain amino

acids from rumen microbial protein synthesis using NH_3 produced in the rumen from blood NO_3 recycled through saliva.

Total ^{15}N excretion in faeces after 141 h in Experiment 1 was much lower than in urine as has been reported in human studies (Wagner, Schultz, Deen, Young & Tannenbaum, 1983; Bednar & Kies, 1994). The recovery of ^{15}N after the intravenous injection of $^{15}NO_3$ in total combined faecal and urine excretion indicated that 64% of the administered ^{15}N dose was excreted after 141 h. The remaining 40% of the administered dose of $^{15}NO_3$ could have been utilised by microorganisms, metabolised, or stored in tissues as suggested by Lewicki et al. (1998). They reported that only 38.5% of the administered $^{15}NO_3$-dose was excreted in urine in the form of $^{15}NO_3$, ^{15}N-urea or ^{15}N-NH_3 after 50 h, without assessing the fate of the remaining 61.5% dose. The possibility of direct NO_3 accretion in the body of ruminants is of high interest since NO_3 reductase activity has been demonstrated in a variety of mammalian tissues and organs (Cohen & Weinhouse, 1971; Eriksson et al., 2018). However the significance of this possible metabolic pathway in ruminants is still uncertain.

5.5.2. Experiment 2

The results obtained in Experiment 2 showed for the first time NO_3 and NO_2 being metabolised and stored in tissues of sheep. A high recovery of ^{15}N-NO_3 and ^{15}N-NO_2, as total ^{15}N present in the skin and muscle of sheep, suggests that these tissues may serve as a pool of NO_3 and NO_2 in the body. Nitrate has been found in human sweat at similar concentrations to in plasma, and because of the acidic conditions of the skin, bacterial nitrate reductases present in the skin generate NO_2 which is rapidly converted to nitric oxide (NO) as reported by Weller et al. (1996). The major cell types that comprise the epidermis, the dermis and hypodermis, including keratinocytes, fibroblasts, melanocytes and endothelial cells, express NO synthase activity and appear capable of releasing NO in humans (Cals-Grierson & Ormerod, 2004). Furthermore, dietary NO_3 has been shown to increase skin temperature and wool growth in sheep, where vasodilation might have increased capillary blood flow and nutrient supply (Li et al., 2013; de Raphélis-Soissan et al., 2014).

Regarding the high recoveries of total ^{15}N in muscle, previous studies in humans and rats have shown that skeletal muscle is the largest body reservoir for NO_3 (Nyakayiru et al., 2017) which is also available for systemic use during dietary NO_3 deprivation. Conversely, NO_2 was more homogeneously distributed among internal organs, blood and skeletal muscle

(Piknova et al., 2015; Gilliard et al., 2018). Mammalian tissues metabolise NO_2 much differently than does blood. It has been reported that NO_2 is present in the heart and liver of rats but is quickly and completely oxidised to NO_3 in blood. This suggests that plasma NO_2 concentrations which are also affected by dietary intake of NO_2 and NO_3, do not accurately reflect NO_2 concentrations in tissues (Bryan et al., 2005; Bryan, 2006)

Keeping in mind the short 60 min observation period after dosing with $^{15}NO_3$ and $^{15}NO_2$ in this study, substantial recovery of total ^{15}N was also evident in the liver and the small intestine. Nitrate sequestration and storage in the liver of rats has been shown in the study of Gilliard et al. (2018). Furthermore, Eriksson et al. (2018) reported that NO_3 was secreted into and accumulated in the small intestinal lumen of anesthetized pigs, locally converted to NO_2, and then absorbed and transported via the portal vein to the liver. Here, NO_2 was taken up and possibly subsequently converted to NO and other bioactive nitrogen oxide compounds.

Between 2.5 and 4% of the ^{15}N administered as intravenous doses of $^{15}NO_3$ and $^{15}NO_2$ were recovered in saliva, and also N in the pancreas and gallbladder bile were labelled. Sudies of humans and rats (Witter, Gatley and Balish, 1979) suggest that NO_3 enters in the intestine from the blood either as part of the biliary or pancreatic secretions, or by direct secretion from the bloodstream. Furthermore, NO_3 has been shown to accumulate in the bile of dogs dosed intravenously with $^{15}NO_2$, indicating endogenous oxidation of NO_2 (Fritsch, de Saint Blanquat & Klein, 1985).

5.5.3. Experiment 3

Appearance of (NO_3+NO_2) in plasma immediately after dosing sheep with NO_3 or NO_2 into the isolated rumen confirms direct absorption of these ions through the rumen wall into the bloodstream (Pfander et al., 1957; Stumpff, 2011). In sheep dosed with NO_3 or NO_2 into the rumen, NO_3+NO_2 was rapidly concentrated in blood plasma and consequently blood MetHb concentrations were increased. However, sheep dosed with NO_3 into the rumen had lower blood MetHb concentrations relative to sheep dosed with NO_2, consistent with NO_2 being more rapidly taken up by blood red cells, and NO_2 being more reactive than NO_3 and having a higher capacity to reduce haemoglobin (Hon, Sun, Dejam & Gladwin, 2010). Concentrations of blood MetHb in sheep dosed with NO_3 or NO_2 into the rumen were below 20% of total Hb, a threshold value reported to be unsafe for ruminants (Bruning-Fann &

Kaneene, 1993). Increasing concentrations of NO_3+NO_2 in saliva after dosing in all sheep indicate NO_3 being concentrated via enterosalivary circulation as reported in humans (Koch et al., 2017).

Results for plasma NO_3, NO_2 and blood MetHb concentrations after dosing sheep with NO_3 or NO_2 into the abomasum and small intestine indicated that both ions were absorbed from these organs. However, evidence of rapid NO_3 absorption, but not NO_2, was observed in the abomasum. It is hypothesised that dosed NO_2 was chemically converted to NO in the acidic conditions of the abomasum, resulting in low blood NO_2. In humans, low pH values in the stomach play an important role in NO_3, NO_2 and NO metabolism via the denitrification pathway (Lundberg, Weitzberg & Gladwin, 2008; Koch et al., 2017; DeMartino, Kim-Shapiro, Patel & Gladwin, 2018). However, blood NO_3 increased following abomasal NO_2 introduction so further metabolism of NO to NO_3 must have occurred in the abomasum.

Greater plasma NO_3 relative to NO_2 concentrations in all sheep indicate that absorbed NO_2 was oxidised to NO_3 in the blood of sheep as has been reported in humans (Bryan & Ivy, 2015). The NO_2 ion has a rapid reactivity with blood Hb and other endogenous compounds that regenerate NO_3 *in vivo*, by oxidative transformation (DeMartino et al., 2018). This NO_3 is then excreted in saliva and urine, explaining the low concentrations of NO_2 relative to NO_3 in those fluids in all sheep. Similar results have been observed in plasma, saliva and bile of dogs, dosed intravenously with $^{15}NO_3$ and $^{15}NO_2$ (Fritsch et al., 1985). More recently, Eriksson et al. (2018) reported that NO_3 is generated and accumulated in the small intestinal lumen in pigs, and locally converted to NO_2 by a mammalian nitrate reductase and/or gut bacteria.

Blood MetHb concentrations after dosing sheep with NO_3 in the abomasum and small intestine were low and stable, relative to sheep dosed into the rumen, suggesting little reduction of NO_3 to NO_2 occurs outside the rumen. Regarding sheep dosed with NO_2 into the small intestine, blood MetHb concentrations were between 1.5 and 5-fold greater than in sheep dosed into the rumen, suggesting a more rapid absorption of NO_2 from the small intestine into the blood. Mirroring the negligible plasma NO_2 concentrations, blood MetHb concentrations were also very low in sheep dosed with NO_2 into the abomasum, although it is noted this sheep received a reduced dose of NO_2 (Table 5.1).

Recoveries of (NO_3+NO_2) in urine in most sheep in Experiment 3 were due to NO_3 excretion (data not shown). The highest urinary recovery was observed in sheep dosed with NO_2 in the small intestine, suggesting rapid absorption and metabolism of NO_2 but not NO_3 in this organ (Tables 5.6 & 5.9). Dosing sheep with NO_2 in the rumen, abomasum or small intestine interrupted urine production. A possible explanation for this result may be the potent vasodilator effect of NO and the consequent reduction in blood pressure, with particular sensitivity to NO_2 in the renal vasculature as reported in humans (Carlström, Lundberg & Weitzberg, 2018).

5.6. Conclusions

Results obtained in the current experiments confirmed that NO_3 and NO_2 were rapidly absorbed through the rumen wall into blood. Greater plasma NO_3 relative to plasma NO_2 concentrations in sheep dosed with NO_3 or NO_2 into the rumen, abomasum or small intestine suggest that NO_2 was oxidized to NO_3 in the bloodstream. Also NO_3 was concentrated into plasma and saliva via enterosalivary circulation. Appearance of ^{15}N as urinary ^{15}N-urea, when administered as $^{15}NO_3$ into the blood, confirms passage of plasma NO_3 to the digestive tract and microbial reduction to ammonia in the rumen or hindgut, conversion to urea in the liver to then be excreted in urine or recycled via saliva into the gut. Apart from reabsorption and NO_3 and NO_2 being rapidly metabolised and recycled into the GIT of sheep, these ions were shown to accumulate in tissues for at least one hour after intravenous injection of ^{15}N-NO_3 and ^{15}N-NO_2, explaining the low urinary excretion and low urinary recoveries of NO_3 and NO_2 observed in the current experiments.

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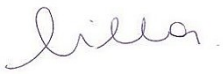

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Chapter 6. The effects of dietary nitrate on plasma glucose and insulin sensitivity in sheep

Title of Article: The effects of dietary nitrate on plasma glucose and insulin sensitivity in sheep.

Authors: M. L. Villar; I. R. Godwin; R. S. Hegarty; R. C. Dobos; K. A. Smith; J. W. Clay and J. V. Nolan.

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The effects of dietary nitrate on plasma glucose and insulin sensitivity in sheep

6.1. Abstract

Nitrate (NO_3) is an effective non-protein nitrogen source for gut microbes and reduces enteric methane (CH_4) production in ruminants. Nitrate is reduced to ammonia by rumen bacteria with nitrite (NO_2) produced as an intermediate. The absorption of NO_2 can cause methaemoglobinaemia in ruminants. Metabolism of NO_3 and NO_2 in blood and animal tissues forms nitric oxide (NO) which has profound physiological effects in ruminants and has been shown to increase glucose uptake and insulin secretion in rodents and humans. We hypothesized that absorption of small quantities of NO_2 resulting from a low-risk dose of dietary NO_3 will increase insulin sensitivity (S_I) and glucose uptake in sheep. We evaluated the effect of feeding sheep with a diet supplemented with 18 g NO_3/kg DM or urea (Ur) isonitrogenously to NO_3, on insulin and glucose dynamics. A glucose tolerance test using an intravenous bolus of 1 mL/kg LW of 24% (w/v) glucose was conducted in twenty sheep, with 10 sheep receiving 1.8% supplementary NO_3 and 10 receiving supplementary urea isonitrogenously to NO_3. The MINMOD model used plasma glucose and insulin concentrations to estimate basal plasma insulin (I_b) and basal glucose (G_b) concentration, insulin sensitivity (S_I), glucose effectiveness (S_G), acute insulin response (AIRg) and disposition index (DI). Nitrate supplementation had no effect ($p > 0.05$) on I_b but shortened the time to peak insulin after glucose injection. The decrease in blood glucose occurred at the same rate (S_G; $p = 0.60$) and there was no effect of NO_3 on either G_b, S_I, AIRg or DI. This experiment found that the insulin dynamics assessed using the MINMOD model were not affected by NO_3 administered to sheep at a low dose of 1.8% NO_3 in the diet.

Key words: MINMOD, ruminants, nitric oxide, nitrite, urea

6.2. Introduction

Dietary NO_3 can replace urea as a non-protein nitrogen source for ruminants consuming low quality diets (Leng, 2008; Nolan, Hegarty, Hegarty, Godwin & Woodgate, 2010) while reducing enteric methane (CH_4) and improving animal productivity (Li et al., 2013; Nguyen, Barnett & Hegarty, 2016). Nitrate is reduced to ammonia in the rumen by the rumen biota and nitrite (NO_2) is generated as an intermediate. The absorption of NO_2 at high levels results in toxemia but gradual introduction of dietary NO_3 allows the rumen microbiota to adapt (van Zijderveld et al., 2010). Inclusion of 1.8% NO_3 in the diet has shown to reduce CH_4 yield (L CH_4/ kg DMI) between 15% and 35% without risking NO_2 toxicity in sheep (Li, Davis, Nolan & Hegarty, 2012) or cattle (Duthie et al., 2016) and maybe a practical level for safe but effective enteric CH_4 mitigation.

Dietary NO_3 and NO_2 are metabolized in blood and tissues to form nitric oxide (NO) and other bioactive nitrogen oxides (Nyström et al., 2012; Wang et al., 2018). Indeed, NO_3 can act as a substrate for NO generation through the obligate anion NO_2 which is further metabolized to NO, via numerous enzymatic as well non-enzymatic pathways in blood and tissues (Lundberg, Weitzberg & Gladwin, 2008). Endogenous NO is produced from L-arginine from the action of NO synthases, and insulin acutely stimulates NO synthesis from arginine (Tessari et al., 2007). Effects of NO on glucose uptake and insulin secretion have been reported in humans, rodents and sheep (Schmidt, Warner, Ishii, Sheng & Murad, 1992; Lundberg et al., 2009; Nyström et al., 2012). There is only one previous experiment that evaluated the effect of NO_2 on glucose tolerance and insulin sensitivity in sheep (Turner, Godwin & Dobos, 2014).

Glucose is the major energy source for the body and insulin, secreted by the β-cells of the islets of Langerhans in the pancreas, is a key regulating agent in energy metabolism, promoting the uptake and utilization of glucose by many peripheral tissues (Ruckebusch, Phaneuf & Dunlop, 1991). Insulin inhibits hepatic glucose production (Girard, 2006) and stimulates glucose utilization in skeletal and cardiac muscle and adipose tissues by activating the translocation of glucose transporters type-4 (Kahn, 1992). Blood flow in the skeletal muscle is stimulated by insulin secretion (Roy, Perreault & Marette, 1998) and the enhancing effect of insulin on the muscle vasculature is mediated by the release of NO by the endothelium (Schmidt et al., 1992; Baron, 1994; Steinberg, Brechtel, Johnson, Fineberg & Baron, 1994; Tessari et al., 2007). Insulin also inhibits the degradation of glycogen and activates its synthesis from glucose in liver and in muscle tissue (Ruckebusch et al., 1991).

The aim of this present experiment was to investigate whether feeding sheep with a diet supplemented with a low dose of 18 g NO_3/kg DM would result in an increase of glucose uptake by increasing insulin release and insulin sensitivity.

6.3. Materials and methods

6.3.1. Animals and diets

The experiment was conducted in compliance with all protocols approved by the Animal Ethics Committee of the University of New England (AEC16-076).

Twenty crossbred sheep (78.7 ± 5.8 kg) were housed in individual pens and allocated by stratified randomization based on live weight to two isonitrogenous dietary treatments. A chaffed blend of lucerne and oaten hays was supplemented with 18 g NO_3/kg DM as calcium nitrate ($5Ca(NO_3)_2$. $NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway) or with inclusion of Ur (9.8 g urea/kg DM). Nitrate or urea supplements were dissolved in water and sprinkled over blended chaff as it was tossed in a ribbon mixer. Sheep were offered the daily ration at 2% of individual live weight (LW) once per day at 0830 hours, and were acclimated to the diet over 21 days. Water was available *ad libitum.*

6.3.2. Blood sample collection and analyses

Glucose and insulin dynamics were measured on Day 22 (5 sheep per dietary treatment) and Day 24 (5 sheep per dietary treatment). Sheep were fasted for 24 h before the intravenous glucose tolerance test (IVGTT) was performed. Polyethylene catheters (60 cm of length, OD 1.50 mm x ID 1.00 mm, Sterihealth, VIC, Australia) were inserted into the jugular vein through a double bevel introducer needle (14G, Surflo I.V. catheter, Philippines) and flushed and filled with 10 IU heparin/mL 0.9 % saline after each sampling and removed after the last blood collection of the day. The pre-warmed (38ºC) bolus dose of 1 mL/kg LW of 24 % (w/v) glucose solution (Sigma-Aldrich, G8270, ≥ 99.5%) was administered intravenously to the opposite jugular vein within 1 min. Blood samples (8 mL) were collected in 10 mL heparinised vacutainers (BD Vacutainer, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) at -30, 0, 2, 4, 8, 15, 20, 30, 45, 60, 90, 120, 180 and 240 mins after administration of glucose and were immediately placed on ice. Blood methaemoglobin (MetHb) concentration was determined in background samples (-30 min) within 15 min of collection by a blood gas analyser (ABL 800 Flex, Radiometer, Brønshøj, Denmark). Blood samples were centrifuged

at 1050*g* x 15 min (4 °C; Beckman TJ-6, USA) with plasma separated by pipette and stored at -20 °C until further analyses.

Plasma NO_3 and NO_2 concentrations were determined in background samples after deproteinisation with 95% ethanol and subsequently analysed in a continuous flow analyser (SAN+ +, Skalar, Breda, Netherlands) as described by de Raphélis-Soissan et al. (2017). For glucose determination, 60 μL of the thawed plasma was subsampled into 1.5 mL sample cups for enzymatic assay and spectrophotometric analysis at 340nm (Dade Behring Dimension Xpand Plus Integrated Chemistry Analyser, Ramsey, Minnesota, USA). Ovine insulin concentration was determined using a competitive sandwich enzyme immunoassay ELISA (Abnova, Taipei, Taiwan R.O.C) with colorimetric detection at 450nm and sensitivity of 0.5 ng/mL (SpectraMax M2e Microplate Reader, Molecular Devices, USA).

6.3.3. Minimal model parameters

The variables of the minimal model of glucose and insulin dynamics were determined from the time series data of plasma, insulin and glucose concentration following the IVGTT, using MINMOD (Boston et al., 2003). Fasted glucose and insulin concentrations were calculated by averaging baseline samples (-30 min and 0 min). Insulin sensitivity (S_I), and glucose effectiveness (S_G, rate of glucose clearance at basal insulin) were determined by simultaneous fitting of the glucose and insulin concentration curves, according to the following equations using MINMOD.

$$G'(t) = -(S_G + X) \times G(t) + (S_G \times Gb) \qquad (1)$$

where G(t) is glucose concentration at minute (t) and Gb is baseline glucose concentration;

$$X'(t) = -P_2 \times X(t) + P_3 \times [I(t) - I_b] \qquad (2)$$

where X(t) is insulin action at minute (t), I(t) is insulin concentration at minute (t), I_b is baseline insulin concentration, P_2 is loss rate of insulin action (X), and P_3 is action of one unit of insulin on glucose disposal per minute.

Insulin sensitivity (S_I) quantifies the capacity of insulin to promote glucose disposal though the GLUT4 receptors and to inhibit the endogenous production of glucose. It is calculated as the acceleration of glucose clearance by insulin ($S_I = P_3/P_2$) (Hoffman, Boston, Stefanovski, Kronfeld & Harris, 2003). Glucose sensitivity (S_G), the capacity of glucose to mediate its own disposal through GLUT1 receptors is calculated as the rate of glucose

clearance at basal insulin (S_G). Accute insulin response (AIRg) measures the adequacy of insulin secretion through β-cell function (defined as the area under the curve between 0 – 10 minutes) following glucose injection available to act on glucose clearance (Hoffman et al., 2003; Long et al., 2010). The disposition index (DI) is an index of the absolute insulin action potential determined by the initial insulin secretion response and the tissue response (DI = AIRg x S_I).

6.3.4. Statistical analyses

Data on glucose and insulin concentrations in plasma were analysed using a linear mixed model *lme* (Pinheiro & Bates, 2011) with repeated measures analysis of variance. Diet and time were considered as fixed effects while sheep and sampling day as random effects. Plasma NO_3 and NO_2 concentrations and blood MetHb concentrations were analysed using a linear mixed effect model considering diet as fixed effect and sampling day as random effect. Two sheep in the NO_3 group and one sheep in the Ur group were excluded from the analysis because of blockage in the catheter at the time of sampling or poor fit of the minimal model to the data. Non-parametric statistical test, Mann-Whitney-Wilcoxon, was used to analyse the parameters Gb, Ib, S_I, S_G AIRg and DI derived from MINMOD (Boston et al., 2003) because distribution of data was non-normal. All statistical tests were carried out in R 3.3.1 (R Core Team, 2016). Homogeneity of variance and normal distribution were tested using residuals plots and Shapiro-Wilk test. Differences were considered as significant at $p < 0.05$ and tendencies when $0.05 < p < 0.10$. Data are reported as mean ± SEM or as median ± interquartile range (IQR, difference between 75^{th} and 25^{th} percentiles). Coefficient of variation (CV) is also specified when required.

6.4. Results

6.4.1. Effect of nitrate supplementation on blood methaemoglobin, plasma nitrate and nitrite concentrations.

Sheep fed both the NO_3 and Ur diets showed low blood MetHb as a proportion of total haemoglobin (p = 0.40; Table 6.1). Plasma NO_3 concentration in samples taken before the glucose injection were greater in sheep fed dietary NO_3 than the Ur diet (p = 0.02) but no differences were observed for plasma NO_2 concentration (p = 0.87). There was a high variability in plasma NO_3 concentrations between sheep fed the NO_3 diet (CV = 74.7%) but this was not observed in the Ur-fed sheep (CV = 22.1%).

Table 6.1. Blood methaemoglobin (MetHb) as a percentage of total haemoglobin and plasma NO_3 and NO_2 concentrations in background samples taken before the intravenous injection of glucose in sheep supplemented with 1.8% nitrate in DM (NO_3) or urea (Ur) isonitrogenous to NO_3 diet.

	Ur	NO_3	SEM	P-value
Blood MetHb (%)	0.95	1.00	0.347	0.40
Plasma NO_3 (µM)	24.50	53.05	6.974	0.02
Plasma NO_2 (µM)	6.13	6.26	0.409	0.87

6.4.2. Effect of nitrate supplementation on glucose and insulin dynamics

Basal concentration of glucose, S_G and I_b were not affected by NO_3 supplementation (p > 0.05; Table 6.2). Dietary NO_3 also had no significant effect on plasma insulin concentrations (p = 0.73; Figure 6.1A) or on glucose tolerance (p = 0.15; Fig 6.1B). The intravenous glucose injection abruptly increased plasma glucose and insulin concentrations (p < 0.001; Figures 6.1A, 6.1B) although no differences were observed in peak insulin concentrations between dietary treatments (NO_3 1.75±0.40 IU *v.* Ur 1.64±0.23 IU; p = 0.94). Variability in plasma insulin concentrations within sheep fed the dietary NO_3 (CV = 41.3%, Figure 6.1A) was greater than the Ur-fed sheep (CV= 35.3%, Figure 6.1B). One sheep fed the NO_3 diet always had a plasma insulin concentration more 1.5-fold greater than the average of the seven other sheep. Plasma glucose concentration (Figure 6.1B) was much less variable than plasma insulin and decreased at the same rate (S_G) in both NO_3 and Ur supplemented sheep (p =

0.60; Table 6.2). No effects of NO_3 supplementation were observed for the parameters S_I, AIRg and D_I ($p > 0.05$, Table 6.2).

Table 6.2. Glucose and insulin MINMOD parameters for sheep fed a blended oaten/lucerne chaff supplemented with 1.8% nitrate in DM (NO_3) or urea (Ur) isonitrogenous to NO_3 diet. Data shown as median and interquartile range (IQR: difference between 75^{th} and 25^{th} percentiles).

MINMOD parameter	Ur (n = 9)		NO_3 (n = 8)		
	Median	IQR	Median	IQR	P-value
Insulin sensitivity (SI), (mU/L)-1.min-1	3.32	0.44, 4.13	3.14	0.08, 7.20	0.80
Glucose effectiveness (SG), min-1	0.04	0.02, 0.06	0.01	0.01, 0.03	0.42
†AIRg, mIU.L-1.min-1	132.51	112.26, 167.96	163.00	54.25, 228.78	0.70
Disposition index (DI)	137.01	48.16, 620.41	271.36	16.77, 737.07	0.60
Basal glucose (Gb), mg.dL-1	73.33	70.61, 79.32	79.84	70.20, 87.38	0.60
Basal insulin (Ib), mU.L-1	24.11	19.08, 28.54	24.63	19.83, 32.27	0.50

†Acute insulin response to glucose

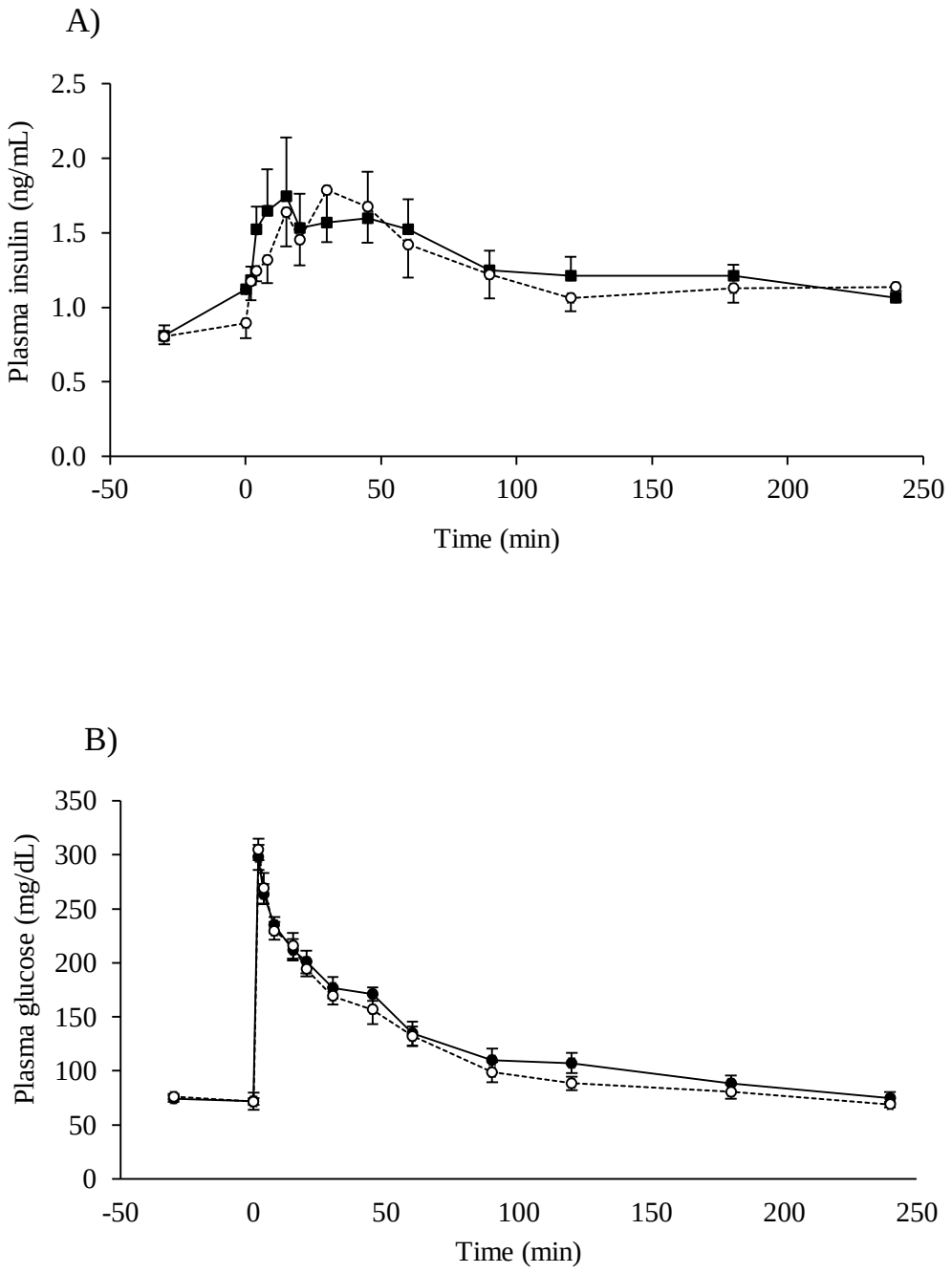

Figure 6.1. Means of plasma insulin (A, ng/mL) and glucose concentrations (B, mg/dL) during an intravenous glucose tolerance test performed in sheep supplemented with 1.8% nitrate in dietary DM (—■—, NO_3; n = 105) or urea isonitrogenous to NO_3 (--○--, Ur; n = 135). Vertical bar indicates SEM.

6.5. Discussion

The effect of dietary NO_3 and NO_2 on insulin and glucose dynamics has predominantly been studied in rodents (Nyström et al., 2012) and humans (Lundberg et al., 2008). Turner et al. (2014) reported an increase in insulin sensitivity determined by intravenous glucose tolerance test (IVGTT), after intravenous infusion of NO_2 in sheep. The goal of the current experiment was to examine the possibility that absorption of small quantities of NO_2 following consumption of a diet with a low-risk dose of dietary NO_3 could lead to an increase in glucose effectiveness and insulin sensitivity in sheep, which may have beneficial effects on nutrient metabolism in ruminants. To test the hypothesis, an IVGTT was performed in sheep and glucose and insulin parameters were estimated using MINMOD (Boston et al., 2003).

Background blood MetHb concentrations were low and within the range for basal MetHb concentration in sheep (Power et al., 2007). Concentrations of blood MetHb and plasma NO_3 and NO_2 in sheep fed the NO_3 diet were in agreement with previous findings using a similar dose of dietary NO_3 (De Raphélis-Soissan, Nolan, Newbold, Godwin & Hegarty, 2016). Low background concentrations of blood MetHb in sheep fed the NO_3 diet were due to their low concentrations of plasma NO_2 arising from the low dietary NO_3 inclusion and due to the 24 h fasting period before sampling.

There was a large variation for the minimal model parameters AIRg and DI within sheep in both dietary treatments and greater variability in S_I was observed between sheep fed the NO_3 diet relative to Ur (Table 6.2). Although no significant differences were observed in the peak value of insulin between dietary treatments, from Figure 6.1A it is apparent that peak value was more rapidly achieved in sheep fed the NO_3 diet than in the Ur-fed sheep. A short time to peak insulin is positively associated with insulin sensitivity in humans (Hayashi et al., 2013) and it has also been suggested that NO may be important in the signal transduction pathway of the early phase of glucose-stimulated insulin secretion (Spinas et al., 1998; Spinas, 1999). However, glucose concentration after intravenous glucose injection was equally reduced to normal levels in both dietary treatments throughout the IVGTT (Figure 6.1B).

The level of NO_3 inclusion in the diet in the current experiment was selected according to previous studies where CH_4 reduction was achievable without risking NO_2

toxicity. Although no effect of NO_3 supplementation on S_G and S_I was observed in this experiment, some evidence of associations of NO_3 or NO_2 and insulin dynamics were reported previously. Nyström et al. (2012) found that plasma NO_2 at a concentration of 10 µM increased insulin secretion in rats but NO_3 was devoid of any insulinotropic action. An increase in glucose tolerance in mice supplemented with a chronic low-dose of $NaNO_3$ (0.1 mmol/kg/d) was reported by Carlström et al. (2010). Turner et al. (2014) found an increase in S_I in sheep after an infusion of 3 mg $NaNO_2$/min during 6.5 hours. The difference in the results for S_I obtained in the current experiment relative to Turner et al. (2014) may be explained by the low concentration of plasma NO_2, which appears to be too low 24 h after feeding to affect glucose and insulin dynamics.

It has been reported that feeding NO_3 to ruminants substantially altered the diurnal pattern of CH_4 production relative to urea fed animals (Nolan et al., 2010; Van Zijderveld et al., 2011; Wang et al., 2018). These authors suggest that NO_3 delays peak of ruminal fermentation which could have affected the blood glucose concentrations and therefore the glucose parameters estimated with MINMOD in this experiment. However, the last meal offered to sheep in this experiment was 24 h before blood samples were taken, while NO_3 effects on rumen fermentation are known to be greatest within 5 to 7 h after feeding NO_3 (Van Zijderveld et al., 2011; Wang et al., 2018).

In this experiment, sheep had been supplemented with a low dose of NO_3, and on the day of the IVGTT had been fasted before sampling, so that blood glucose concentration could stabilize. Fasting and the modest dose of dietary NO_3 may have limited the expected effects of NO_3 on glucose disposal and insulin sensitivity, as suggested by James, Willis, Allen, Winyard and Jones (2015) and Gilchrist et al. (2014). However, plasma NO_3 was greater in sheep fed the NO_3 diet relative to Ur-fed sheep, although low concentrations of NO_2 were recorded in all plasma samples without affecting S_I. Nevertheless, increasing the dose of NO_3 in order to moderate blood glucose kinetics may be risky, as sheep might be exposed to NO_2 toxicity.

Ingestion of dietary NO_3 affects plasma glucose and insulin concentrations in humans, even at low concentrations (James et al., 2015). Changes in circulating concentrations of NO_3, NO_2 or NO following NO_3 therapy, may be dependent on acute or

chronic administration of NO_3 (Carlström et al., 2010; Khalifi et al., 2015). This experiment equated to chronic administration of NO_2 showed no effect of dietary NO_3 on S_I, although S_I concentrations were within the same range as those obtained by Turner et al. (2014) after infusing sheep with NO_2. Compared with their results, there was high variability in plasma insulin concentrations within this experiment the NO_3 fed sheep and smaller values for glucose effectiveness were obtained.

6.6. Conclusions

The results obtained from this research suggest that NO_3 supplementation administered at a low dose of 1.8% NO_3 in dietary DM did not affect glucose uptake or insulin sensitivity in sheep. However, the large between-sheep variability observed in insulin concentrations in sheep fed the NO_3 diet suggests a possible effect of dietary NO_3 on insulin metabolism. Future research on between-animal variation in NO_3 metabolism and the effect of different levels of NO_3 supplementation on insulin sensitivity is recommended.

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The effects of dietary nitrate on plasma glucose and insulin sensitivity in sheep

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We, the Research 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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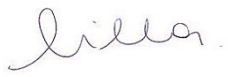

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Chapter 7. General discussion

General discussion

7.1. Introduction

Nutritionally, the inorganic anions NO_3 and NO_2 have been considered inert and unfavourable dietary constituents for ruminants and end products of nitric oxide (NO) metabolism with potentially carcinogenic effects in humans. However, in the last 10 years research has shown that NO_2 and NO_3 can prevent and mitigate many diseases and improve physical performance (Lundberg, Weitzberg & Gladwin, 2008; Bryan & Ivy, 2015). Similarly, research on dietary NO_3 in livestock production was originally focused on the risk of NO_2 toxicity as a consequence of the presence of NO_3 in pastures and drinking water (EFSA, 2009). But recently, focus has been on the effectiveness of dietary NO_3 in reducing enteric CH_4 production from ruminants (Lee & Beauchemin, 2014). Some findings suggesting favourable physiological effects of NO_3 metabolites in livestock production (Cottrell, Ponnampalam, Dunshea & Warner, 2015). Options to manage the rumen to limit the incidence and severity of NO_2 poisoning have been studied (Nolan, Godwin, de Raphélis-Soissan & Hegarty, 2016) as has the efficacy of combining NO_3 with vegetable oils to reduce enteric CH_4 production (Duthie et al., 2018). The absorption of NO_3 and NO_2 has been more widely studied in non-ruminants (Friedman, Greene & Epstein, 1972; Koch et al., 2017; Eriksson, Yang, Carlström & Weitzberg, 2018) and was poorly defined in ruminants.

Nitrate reduction in mammals largely requires enteric symbiotic bacteria, generating NO_2, which is then further reduced to nitric oxide (NO) in the acid stomach or absorbed into the circulation where it is reduced to NO by haemoglobin or the molybdopterin family of enzymes (DeMartino, Kim-Shapiro, Patel & Gladwin, 2018). Approximately 25% of total circulating NO_3 in mammals is actively sequestered into salivary glands, being concentrated in saliva up to 20 times that in plasma and reduced to NO_2 by oral bacteria (Witter, Balish & Gatley, 1979; Koch et al., 2017). Once swallowed, NO_2 undergoes a diversity of downstream reductions, including formation of NO and NO-donating species (Sobko et al., 2005; Koch et al., 2017). In ruminants, it is widely accepted that NO_3 and NO_2 are directly absorbed through the rumen wall of ruminants; without clear evidence in literature (Pfander, Garner, Ellis & Muhrer, 1957; Stumpff, 2011). Nitrate recycling via saliva to the digestive tract circulation has been suggested since appearance of ^{15}N in urinary urea-N was found

and continued to increase 6 h after post intravenous injection of ^{15}N-NO_3 in sheep (Lewicki, Wiechetek, Souffrant, Karlik & Garwacki, 1998). More information about the absorption sites and kinetics of absorption of NO_3 metabolites in ruminants is crucial to improve the safety of using dietary NO_3 to reduce GHG emissions from livestock production. Furthermore, large between-animal variation in blood MetHb levels after ingesting diets supplemented with NO_3 has been reported (Cockrum, Austin, Kim, et al., 2010), suggesting differential NO_3 metabolism within animals, probably including different extents of metabolism by the rumen biota.

The principal objective of this thesis was to improve understanding of the basic biology of NO_3 absorption and NO_2 formation in ruminants in order to increase the safety of NO_3 supplementation (Chapter 5). Further, we sought to evaluate the contribution of ruminal protozoa to NO_3 metabolism in ruminants, specifically quantifying their role in NO_3 and NO_2 reduction in the rumen (Chapter 4). This program also investigated the effect of dietary NO_3 on animal performance in diets containing two contrasting levels of CP, and whether the combination of NO_3 and canola oil reduces enteric CH_4 production (Chapters 2 & 3). Finally, potential beneficial effects of dietary NO_3 and consequently NO on insulin sensitivity and glucose tolerance in sheep were examined in Chapter 6, as indicators of NO_3 affecting major metabolic regulators.

In the following sections the major outcomes of this thesis are discussed highlighting the new knowledge about absorption, partitioning and excretion of NO_3 and NO_2 that will contribute to the understanding and safety of NO_3 supplementation. In addition, sources of between-sheep variability in response to NO_3 supplementation are identified in regard to the results obtained throughout the thesis. Finally, practical implications about the role of NO_3 as a NPN supplement relative to urea is also discussed.

7.2. Implications of nitrate supplementation for rumen fermentation and reducing enteric methane emissions

Traditionally, enteric CH_4 production was considered an inefficiency in ruminant livestock production due to the loss of dietary energy embodied in CH_4. This is still the case but more recent attention has focused on enteric CH_4 as a GHG released to the atmosphere. Livestock contribute to global climate change primarily through CH_4 emissions and practical mitigation strategies are necessary. We discussed in Chapter 1 the effectiveness of the dietary NO_3 as a means of reducing enteric CH_4 without lowering animal production. However, NO_2 toxicity is the main drawback for NO_3 supplementation and therefore maintaining animal health through low blood MetHb concentrations is critical (Chapter 1).

Dietary NO_3 consistently reduced enteric CH_4 production in this thesis and in the literature (Table 7.1). In the rumen, NO_3 acts as an alternative H_2 sink to CO_2 reduction, but also NO_3 and NO_2 inhibit the growth of rumen microorganisms, including protozoa that produce hydrogen, the main substrate for CH_4 formation (Table 7.1). The lower CH_4 production observed in Chapter 2 might also be a consequence of the shorter rumen particulate MRT in NO_3-fed ruminants (Pinares-Patiño et al., 2011; Goopy et al., 2014). Importantly, dietary NO_3 was also effective on reducing CH_4 emissions in a protein-deficient diet (Chapter 3) which has substantial practical implications for tropical areas since NO_3 can replace urea as an NPN supplement (Leng & Preston, 2010). In this case, CH_4 reduction was not associated with NO_3 effects on digesta kinetics but the effect of NO_3 on reducing the protozoa population was substantial (Chapter 3; Table 7.1).

Table 7.1. Effect of dietary nitrate (18 g NO_3/kgDM) on ruminal total volatile fatty acids (VFA), rumen ammonia concentration (NH_3), daily methane production (DMP), methane yield (MY) and total protozoa population expressed as a proportion of the control diet without NO_3 (%). Data shown correspond to results from this thesis and previous studies in sheep or cattle fed with similar doses of dietary NO_3.

	Total VFA	Acetate	Propionate	Butyrate	Acetate/ propionate	NH_3	DMP	MY	Total Protozoa
Chapter 2	-5	+8*	-6	-24*	+16*	+36*	-11*	-9	-33*
Chapter 3	-13	+6	-6	-42*	+15	+359*	-15	-26*	-49*
Average	-9	+7	-6	-33	+16	+248	-13	-17	-41
				Review of literature					
Wang et al (2018)†	-2	-1	+6*	-1	-7*	-34*	-15*	-15*	-1
Nguyen et al (2016)	+39*	+4	-14	-1	+22	+274*	+72*	-25	-26
de Raphelis-Soissan et al (2014)†	+4	+8*	-21*	+4	+36*	-12	-19*	-16*	na
Li et al (2012)†	-19	+16*	-8	-39	+25	na	-34	-35*	-56
Nolan et al (2010)‡	+18*	-7	-19*	-14	+33	+13	na	-13*	-31

*Significant effect of dietary NO_3 ($P < 0.05$); †control diet contained urea (isonitrogenous to NO_3 diet); ‡dose: 25g NO_3/kgDM

Adding NO_3 to the diet of ruminants slightly increases production of nitrous oxide (N_2O), a GHG with a global warming more than 10 times that of CH_4 (Myhre et al., 2013). Nitrous oxide in ruminants is mainly released via eructation (Petersen et al., 2015) and is also produced after N excretion in urine and faeces via microbial denitrification in the soil. Recent *in vivo* and *in vitro* experiments reported that adding NO_3 to the diet of ruminants increased enteric production of N_2O (de Raphélis-Soissan et al., 2014; de Raphélis-Soissan et al., 2017). Further calculations using data from Chapter 3 showed that NO_3 addition to the diet of sheep reduced the net GHG mitigation attributed to CH_4 mitigation (266 *v* 251 gCO_2-eq/day) by 6% due to an increase in N excretion in urine and faeces (Table 7.2). However, although not statistically significant, sheep fed a diet supplemented with urea had greater urinary urea excretion relative to sheep fed the NO_3 diet (Chapter 3). Considering that urea excreted in urine is the major source of NH_3 volatilised from manure, replacing dietary urea with NO_3 would reduce NH_3 emissions from manure (Lee & Beauchemin, 2014).

Table 7.2. Nitrogen (N) intake, urinary and fecal N excretion and greenhouse gas (GHG) production as methane (CH_4) and nitrous oxide (N_2O) in sheep fed a control diet of wheaten chaff and wheat grain (CON) supplemented with nitrate (18 g/kg DM; NO_3), urea (9.8 g/kg DM;Ur) or cysteamine hydrochloride (80 mg/kg liveweight; CSH)

Parameter	CON	NO_3	Ur	CSH	s.e.m	*P*-value
N intake (g N/day)	3.2a	5.2b	5.2b	3.8a	0.30	<0.01
Urinary N (g N/day)	3.0a	4.8b	4.3b	2.8a	0.24	<0.01
Faecal N (g N/day)	2.5	2.7	2.7	2.6	0.12	0.96
DMP (g CH_4/d)	10.3	8.8	9.2	10.9	0.57	0.31
N_2O from manure (mg/d)[A]	59.0	71.2	66.6	54.1	2.51	0.06
GHG as CH_4 (g CO_2-eq/d)	256.5	219.6	237.8	273.3	13.50	0.27
GHG as N_2O (g CO_2-eq/d)	17.6	21.2	19.8	16.1	0.75	0.05
Total GHG (g CO_2-eq/day)	266.2	250.6	299.7	289.4	12.47	0.49

[A]Value of 1% for the N_2O emission factor for sheep excreta (IPCC, 2006)

GHG: greenhouse gas based on assumed global warming potentials of 25 CO_2-eq for methane and 298 CO_2-eq for nitrous oxide (Forster et al. 2007)

Petersen et al. (2015) reported that N_2O eructated from dairy cattle fed a diet supplemented with NO_3 was the major contributor to N_2O emissions relative to urinary and faecal N_2O emissions, suggesting that denitrification may occur in the rumen and the oral cavity of ruminants. Although N_2O emissions may decrease the net GHG mitigation effect

of dietary NO_3, the regulatory mechanism of denitrification in the rumen in not yet fully understood.

Despite the benefits of reducing enteric CH_4 in NO_3-fed animals, some adverse effects of NO_3 supplementation on DMI and DMD were observed in this thesis (Chapter 2). As we discussed in Chapter 1, NO_3 may affect palatability and thus may reduce DMI, however results available in the literature are contradictory and in some cases feeding NO_3 did not affect, or even improved feed intake and feed digestibility (Table 1.2). Additionally, we observed that including NO_3 in a protein-deficient diet fed to sheep, had potential to reduce enteric CH_4 without affecting DMI or DMD (Chapter 3). Similarly, other studies that evaluated low-protein diets supplemented with NO_3 reported a reduction in MY with an increase in DMI, DMD or animal performance (Nguyen, Barnett & Hegarty, 2016; Tomkins, Parker, Hepworth & Callaghan, 2016). However, a previous meta-analysis showed that across diets there is no relationship between NO_3 level in the diet and DMI (Lee & Beauchemin, 2014).

In general, the results of this thesis showed that feeding NO_3 did not affect total VFA concentration in the rumen fluid (Table 7.1). The most consistent effect of dietary NO_3 was a reduction in the proportions of butyrate in the rumen fluid (Chapters 2 & 3), there was also an increase in acetate proportion and therefore the AP ratio was greater (Chapter 2). The results observed in this thesis confirm that the effects of dietary NO_3 on total VFA concentration and molar proportions of VFA are not consistent as evident within the literature (Table 7.1).

Overall, adding NO_3 to the diet of ruminants increased ruminal NH_3 concentration relative to diets without NO_3 as observed throughout this thesis and in the literature (Table 7.1), confirming that NO_3 is a source of fermentable-N for microbial growth. However, results from Chapter 3 indicated that dietary NO_3 was effective in reducing CH_4 emissions in a low protein diet (6.6% CP) but did not show significant effects on N balance relative to diets supplemented with urea or without NPN supplementation. Nevertheless, evidence of NO_3 recycling via saliva to the gut, as is urea, was found in Chapter 3 which is highly beneficial in ruminants fed N-deficient diets and providing additional NO_3-N for anabolic purposes in the rumen such as microbial protein synthesis. Nguyen et al. (2016) reported greater microbial N outflow and an increase in average daily gain in lambs fed a low protein chaff supplemented with 1.9% NO_3 in DM relative to a diet without NO_3 supplementation.

More recently, Wang et al. (2018) reported greater NH_3 incorporation into microbial protein synthesis and an increase in milk yield in dairy cows fed a low-protein diet supplemented with NO_3 relative to urea supplementation. However, results from Chapter 2 showed that cattle fed a protein-adequate diet (140 g CP/kg DM) supplemented with 18 g dietary NO_3/kg DM did not improve microbial N outflow from the rumen or microbial growth efficiency.

Diets with high NPN to fermentable energy ratios can generate an excess of ruminal NH_3 (> 200 mg N/L) that leads to high rates of absorption of NH_3 and consequently the synthesis of urea in the liver that is then excreted in urine or recycled in the gut. Hepatic synthesis of urea requires energy that may reduce ME available for animal production (Martin & Blaxter, 1965). Regarding the production benefits from CH_4 mitigation, NO_3 supplementation in ruminants seems to be most advantageous in protein-deficient diets, as NH_3 utilization may redirect more N, electrons and energy towards microbial protein synthesis at the expense of CH_4 emissions (Wang et al., 2018).

7.3. Novel aspects of nitrate absorption, partitioning and excretion in ruminants

7.3.1. Nitrate and nitrite absorption from the gastrointestinal tract

As mentioned in Chapter 1, the assumption that NO_2 and NO_3 are directly absorbed through the rumen wall was based on the lack of a noticeable lag before the appearance of MetHb in blood when NO_3 and NO_2 were administered to the rumen; however, clear evidence of this is limited in the literature (Pfander et al., 1957; Stumpff, 2011; Nolan et al., 2016). Results obtained in Chapter 5 indicated that NO_3 and NO_2 were directly absorbed from the isolated rumen into the bloodstream of anaesthetised sheep as NO_3 and NO_2 concentrations in plasma increased immediately after dosing sheep with NO_3 or NO_2 into the rumen. In addition, an indication of NO_2 being oxidised to NO_3 in the blood was also found. Additionally, both NO_3 and NO_2 were rapidly absorbed from the isolated small intestine (Chapter 5), with NO_2 being more rapidly absorbed and consequently producing the highest value of blood MetHb concentration observed in this thesis (48% of total Hb). Although NO_3 was rapidly absorbed from the isolated abomasum, NO_2 was not noticeable in the abomasum contents as it may have been converted to NO due to acidic conditions, mimicking NO_2 metabolism in the stomach of humans (Lundberg & Govoni, 2004).

7.3.2. Nitrate-N recycling, excretion and storage in tissues

Nitrate recycling via enterosalivary circulation was evident from both the *in vivo* experiment (Chapter 3) and anaesthetised sheep experiment (Chapter 5). Nitrate was absorbed from the digestive tract, entered into the bloodstream and concentrated in saliva in the same way as in humans (Koch et al., 2017). Furthermore, the 58% of $^{15}NO_3$-N dosed intravenously recovered as urinary ^{15}N including ^{15}N-urea *in vivo* after 46 h (Chapter 5) confirms passage of NO_3 from plasma to the GIT where NO_3 is reduced to NH_3 by rumen microorganisms, absorbed and converted to urea in the liver and excreted in urine as urea concurring with previous findings (Lewicki et al., 1998). There is no known mammalian mechanism by which NO_3 can be reduced to NH_3. Nitrate recycling through absorption from the GIT and concentration into saliva is highly beneficial in ruminants consuming diets low in protein, providing a continuous supply of fermentable-N for microbial growth via saliva and would have contributed to the improved intake and performance observed in Nguyen et al. (2016).

Regarding results from anaesthetised sheep (Chapter 5, Experiment 3), there was a noticeable lag before ion appearance in urinary or salivary NO_3 or NO_2 when sheep were dosed in the rumen, abomasum or small intestine. However, NO_3 concentrations in urine were consistently greater than urinary NO_2 concentrations even if sheep were dosed with NO_2 in the GIT, reflecting NO_2 being oxidised to NO_3 in the blood. Nitrate concentration into saliva and excretion in urine by all sheep confirm enterosalivary recycling of NO_3 in ruminants. Less than 1% of NO_3 and NO_2 administered dose into the small intestine and abomasum was excreted in urine 180 min after dosing (Chapter 5, Experiment 3).

In vivo results from Chapter 5 (Experiment 1) showed that 64% of the intravenous dose of ^{15}N-NO_3 was recovered in urine and faeces 6 d after dosing suggesting that the remaining 42% of the dose may have been mainly metabolised by rumen microorganisms to produce amino acids which are then absorbed in the intestine and stored in animal tissues (Hutton & Annison, 1972). This is comparable to the only one previous experiment that reported only 40% of the administered $^{15}NO_3$ was recovered in urine as $^{15}NO_3$, ^{15}N-urea and ^{15}N-ammonia 50 h after intravenous injection (Lewicki et al., 1998). Evidence of NO_3-N and NO_2-N storage in tissues was found in Chapter 5 after intravenous injection of $^{15}NO_3$-N or $^{15}NO_2$-N (Experiment 2.2). Appearance of total ^{15}N in skin, muscle and liver 60 min

after dosing suggests that NO_3-N and NO_2-N are stored in tissues of ruminants as has been reported in rats (Piknova et al., 2015) and pigs (Eriksson et al., 2018).

7.4. Variation in ruminal and animal physiology in response to dietary nitrate.

7.4.1. Nitrate supplementation, blood methaemoglobin and animal health

Feeding NO_3 leads to increased blood MetHb levels and may cause methaemoglobinaemia once NO_2 is absorbed into the bloodstream. An increase in plasma NO_3, NO_2 and blood MetHb levels after feeding NO_3 were consistently observed throughout the experiments of this thesis (Table 7.3). Nevertheless, no visible signs of methaemoglobinaemia were observed; suggesting that NO_3 is safe to feed at 1.8% NO_3 in DM when a dietary adaptation period of 15 days is included. Some authors have speculated about the value of inclusion of an adaption period as blood MetHb has been shown to increase over time in sheep and cattle (de Raphélis-Soissan et al., 2014; Godwin et al., 2015). Results from Chapter 4 and from the literature confirm that NO_3 reduction rate increased with exposure to NO_3 indicating an adaptational response by the rumen microbiota (Alaboudi & Jones, 1985; Lin, Schaefer, Guo, Ren & Meng, 2011). However, de Raphélis-Soissan, Nolan, Newbold, Godwin and Hegarty (2016a) in an *in vitro* study showed an increased accumulation of NO_2 in the incubation medium when donor sheep were adapted to dietary NO_3 (20 g NO_3/kg DM) and suggested that adaptation favoured NO_3 reduction more than NO_2 reduction and so could increase risk of NO_2 toxicity.

The toxic dose of NO_3 as reported in the literature is extremely variable and cattle appear to be more susceptible to NO_3 poisoning than sheep, with some individuals more susceptible than others (Leng, 2008). Blood MetHb concentrations between 20-50% may cause cyanosis in ruminants (Bruning-Fann & Kaneene, 1993). The 22.7% of blood MetHb observed in one sheep in Chapter 3 after feeding 1.8% NO_3 in DM could have been a consequence of the pattern of feed intake as the NO_3 diet was offered once daily. Larger meals after a period of fastening increase the peak concentrations of NO_2 in the rumen and more NO_2 will be absorbed into the bloodstream (Benu et al., 2016; De Raphélis-Soissan, Nolan, Newbold, Godwin & Hegarty, 2016b). Furthermore, the particular individual sheep with the highest blood MetHb concentration had blood MetHb concentrations consistently

higher throughout the experiment (Chapter 3). As discussed in Chapter 1, individual animals differ in their tolerance to dietary NO_3 (Cockrum, Austin, Ludden & Cammack, 2010) contributing to the risk of variation in NO_2 toxicity.

When dietary NO_3 was fed in combination with canola oil, the CH_4-mitigating effect was more than additive and without exposing animals to NO_2 poisoning. This combination strategy of dietary NO_3 and lipids offers an opportunity to reduce the amount of NO_3 included in the diet of ruminants and increases the safety of NO_3 supplementation, while reducing CH_4 emissions (Guyader et al., 2015; Duthie et al., 2018). Although several alternatives to reduce NO_2 poisoning associated with NO_3 supplementation have been evaluated (Nolan et al., 2016; De Raphélis-Soissan, 2017), further work investigating the causes of between-animal variability needs to be considered, in order to reduce the risk of methaemoglobinaemia in livestock production.

7.4.2. Rumen microorganisms and between-animal variation in nitrate metabolism

Outcomes from Chapter 3, 4 & 6 indicate that individual animal responses to NO_3 supplementation are highly variable in terms of blood MetHb and concentrations of NO_3 and NO_2 in plasma and rumen fluid (Table 7.3). These results are consistent with previous findings about individual variation regarding tolerance to dietary NO_3 (Cockrum, Austin, Kim, et al., 2010). Furthermore, we also observed a large variation in plasma insulin in sheep fed a diet supplemented with NO_3 that was not evident in the urea-fed sheep (Chapter 6). Previous studies have highlighted the individual variability as a challenge for NO_3 supplementation making NO_2 toxicity unpredictable (Chapter 1).

Table 7.3 Range of plasma NO_3, NO_2 and blood methaemoglobin (MetHb) concentrations (as % of total haemoglobin) in sheep fed 1.8% NO_3 in DM.

	Plasma NO_3 (umol/L)	Plasma NO_2 (umol/L)	Blood MetHb (%)
Chapter 3 (pre and post feeding)	29 - 376	4 - 50	1 - 22.7
Chapter 4 (faunated sheep, pre and post feeding)	80 - 1232	1 - 12	1 - 5.8
Chapter 5 (pre-feeding only)	10 - 40	0 - 10	1 - 1.3
Chapter 6 (pre-feeding only)	25 - 163	4 - 10	0.7 - 1.4
Average	36 - 453	2 - 18	0.9 - 7.8

7.4.2.1. Rumen microbiota

Rumen bacteria are generally responsible for the reduction of NO_3 and NO_2 in the mixed ruminal community. Whether differences in the rumen microbial population are partially responsible for between-animal variation regarding NO_3 metabolism (Veneman et al., 2015) and blood MetHb formation has not been proven yet, but differences in tolerance to dietary NO_3 have been shown in sheep (Cockrum, Austin, Ludden, et al., 2010). As an illustration, in Chapter 3 there was a large variation in blood MetHb concentrations in sheep fed a diet supplemented with NO_3 (CV = 96%) relative to sheep fed diets without NO_3 supplementation (CV = 19%). Within six sheep fed 18 g NO_3/kg DM, blood MetHb concentrations ranged between 1 and 22.7% of total haemoglobin, with one sheep consistently having the highest blood MetHb concentration while another sheep always had the lowest MetHb concentration of the group.

Nitrate and NO_2 reductase activity in rumen bacteria increases in the presence of NO_3 and when ruminants are adapted to NO_3 (Chapter 1). Results from Chapter 4 indicate that *in vivo* and *in vitro* reduction of NO_3 and NO_2 in sheep adapted to dietary NO_3 was stimulated by the presence of NO_3 and rumen protozoa. However, ruminal concentrations of NO_3 and NO_2 *in vivo* after feeding dietary NO_3 and initial *in vitro* concentrations NO_2 after dosing rumen digesta with NO_2 were lower than expected, suggesting possible storage or sequestration of NO_3 by rumen microorganisms and/or NO_2 being bound to proteins as Leng (2008) suggested to occur. More research on NO_3 sequestration or storage by rumen microorganisms needs to be undertaken to clearly understand the association of rumen biota and individual variability to NO_3 metabolism in ruminants.

7.4.2.2. Rumen protozoa and nitrate metabolism

Although rumen microbial populations can adapt to dietary NO_3 (Lee & Beauchemin, 2014), under certain conditions (> 5mM NO_2 in rumen fluid) NO_2 becomes toxic towards rumen bacteria and protozoa (Chapter 1). The analysis of the results from Chapter 2 & 3 indicate that dietary NO_3 reduced rumen protozoa populations in both studies (Table 7.1) which may have contributed to NO_3 reducing enteric CH_4 production. Nguyen et al. (2016) reported that NO_3 in combination with defaunation additively decreased CH_4 production and increased DM intake in sheep fed a protein-deficient chaff supplemented with NO_3.

Ciliate protozoa in the rumen seem to play an important role in NO_3 reduction because of their greater ability to reduce NO_3 than rumen bacteria (Chapter 1). Observations from Chapter 4, like those from Nakamura and Yoshida (1991) and Lin et al. (2011), indicated that NO_3 and NO_2 are more rapidly metabolized in the presence of protozoa as suggested by low ruminal NO_2 concentrations and greater NH_3 production compared to defaunated sheep. Nitrate reduction to NH_3 appeared to be greater in faunated relative to defaunated rumen digesta which may explain the greater accumulation of NO_2 observed *in vitro* (Chapter 4) in defaunated digesta. Some authors attribute this effect to the hydrogenosome present in protozoa that produces hydrogen and release electrons which may stimulate NO_3 reductase activity of bacteria attached to protozoa (Alaboudi & Jones, 1985; Yang, Rooke, Cabeza & Wallace, 2016). Results from Chapter 4 confirmed that reduction of NO_3 is accelerated by the presence of protozoa in the rumen fluid as has been reported previously (Yoshida, Nakamura & Nakamura, 1982).

It is worth considering why recoveries of NO_3 and NO_2 in the rumen digesta in Chapter 4 were less than expected. Apparently, the rapid rates of NO_3 and NO_2 removal from the rumen after ingesting dietary NO_3 is the main explanation for this discrepancy in the recovery of NO_3 and NO_2, but the reasons remain uncertain (Lewis, 1951; Alaboudi & Jones, 1985; Takahashi & Young, 1991; Sar et al., 2004). According to Leng (2008) this inconsistency in the literature may be due to the following possibilities. Firstly, a rapid bacterial metabolism of NO_3 to NH_3 or that NO_3 is stored as amino N to support growth in a more prolonged period of time. Storage of NO_3 has not been studied in the rumen but there is some evidence of occurrence in aquatic ecosystems (Sayama, 2001; Kamp et al., 2018). Secondly, the possibility of NO_3 being temporarily sequestered in rumen microorganisms or that NO_2 might be bound by proteins in the rumen. The third possibility might be that large amounts of NO_3 and NO_2 are rapidly absorbed directly across the rumen wall, as was observed in Chapter 5.

7.4.3. Dietary nitrate and nitric oxide effects on animal physiology

As reviewed in Chapter 1, dietary NO_3 is a source of NO in the body and NO has profound physiological effects reported in humans, pigs and rodents (Lundberg et al., 2008; Gilchrist, Winyard & Benjamin, 2010). The increase of insulin sensitivity is one of the major effects of NO proven in humans relating to the global increase of type 2 diabetes (Ogurtsova et al.,

2017). Understanding insulin action in ruminants is also important for maximising animal production and meat quality. Insulin regulates glycogen synthesis and degradation, with glycogen level in muscle at slaughter being the major determinant of final meat quality (Tarrant, 1989).

The experiment conducted in Chapter 6 aimed to build on a previous study which reported an increase in insulin sensitivity after an acute intravenous NO_2 infusion in sheep (Turner, Godwin & Dobos, 2014). Regarding consequences of feeding dietary NO_3 on insulin and glucose metabolism in sheep, results analysed in Chapter 6 indicate that the NO_3 diet increased circulating plasma NO_3 but insulin sensitivity (S_I) and glucose effectiveness (Sg) were not affected. It seems possible that, concentrations of NO_3 and NO_2 in plasma were not sufficient to produce the level of NO required to trigger insulin production, as sheep were in a fasting condition, as required for glucose tolerance testing. However, there was a greater individual variability in plasma NO_3 and NO_2 concentrations and insulin levels within the NO_3-fed sheep than in the urea-fed sheep. This variability, suggests a possible effect of dietary NO_3, and therefore NO, on glucose and insulin metabolism; adding another component to the between-animal variation in MetHb concentrations discussed previously. In future investigations about the effect of dietary NO_3 on S_I, it might be possible to generate a higher level of circulating NO_2 by intravenous infussion, while undertaking the intravenous glucose tolerance test; although this could be risky in terms of NO_2 toxicity.

Nitric oxide is a potent vasodilator of blood vessels and the formation of NO in response to heat stress has been reported previously (Chapter 1). One of the effects of climate change on livestock production will be mediated through changes in ambient temperatures and the occurrence of heat stress (Thornton, van de Steeg, Notenbaert & Herrero, 2009). As discussed in Chapter 1, dietary NO_3 is a precursor of NO and vasodilation is the physiological reaction of heated-stressed animals. Furthermore, NO synthase responds rapidly to changes in temperature in the body increasing NO production and having a direct effects in vasculature (Cals-Grierson & Ormerod, 2004). Outcomes from Chapter 5 revealed that the highest recovery of ^{15}N in sheep dosed with both $^{15}NO_3$ and $^{15}NO_2$ was found in the skin, suggesting that this tissue may act as a reservoir of NO_3 and NO_2 which may be readily available for NO production. Although a previous study in cattle supplemented with 1% NO_3 did not show effects on the amelioration of heat stress (Simanungkalit, 2015), future work on the current topic is recommended using different levels of dietary NO_3 in ruminants.

The *in vitro* study conducted with rumen fluid from faunated and defaunated sheep incubated with NO_2 (Chapter 4) showed that recovery of NO_2 in rumen fluid was very low, even in the samples dosed with NO_2 and immediately placed in ice to stop microbial activity. This results suggest an instantaneous 'loss' of NO_2 and/or the possibility of NO_2 being bound together with particles in the rumen fluid. The potential formation of NO in the rumen from dietary NO_3 as an alternative means of NO_2 disposal and possible interactions with rumen microorganisms need to be considered in further investigations of ruminal NO_2 metabolism. Studies are also needed to determine whether the effects of NO produced in the rumen are detrimental or beneficial to animal production.

7.5. Replacement of urea by nitrate as a source of non-protein nitrogen to ruminants

Like urea, NO_3 is converted to NH_3 by rumen microorganisms (Lewis, 1951) and it is a source of NPN for microbial growth on diets low in CP. However, both urea and NO_3 may lead to toxicity if rumen NH_3 production from urea exceeds the rate of microbial assimilation or if the absorption of NO_2 from NO_3 exceeds the capacity of blood cells to oxidize NO_2 to NO_3, producing blood MetHb. An adaptation period may help ruminants to adapt to NO_3 or high urea intakes (Alaboudi & Jones, 1985; Leng, 2008); however, on high protein diets an excess of NH_3 production in the rumen might still occur. Ammonia production from NO_3 reduction is slow compared to urea hydrolysis and more efficiently incorporated into ruminal microbial growth (de Raphélis-Soissan et al., 2016a; Lee, Araujo, Koenig & Beauchemin, 2017; Wang et al., 2018). While advantageous for N deficient diets, additional NH_3 production from NO_3 may not be nutritionally beneficial in N-adequate diets (Guyader et al., 2015; Olijhoek et al., 2016).

Replacing urea with NO_3 for the purpose of CH_4 reduction is attractive to industry because NPN supplementation is routine practice; although the cost of NO_3-N supplementation is approximately double the cost of urea supplementation (Callaghan, Tomkins, Benu & Parker, 2014). To compensate the extra cost of NO_3 supplementation, greater evidence of improved animal productivity is needed. However, most studies replacing urea by NO_3 as a source of NPN in N-adequate diets have not enhanced the productivity of ruminants (Lee & Beauchemin, 2014; Table 1.2). A reduction in voluntary

intake in sheep and cattle has sometimes been reported (Table 1.2) and it was also speculated that NO_3 could be used as an intake restrictor in a mixed urea/NO_3 supplement to limit voluntary intake of highly palatable supplements supplied *ad libitum* (Goopy & Hegarty, 2018). Another proposed strategy in the literature is using NO_3 as an alkali pre-treatment of straw and other agricultural by-products low in N to increase the digestibility of the straws while reducing CH_4 production at a lower cost (Zhang et al., 2018).

The beneficial effects of NO_3 supplementation on animal productivity seems to offer greatest opportunity in low-protein diets, although results in Chapter 3 were unable to demonstrate an improvement in animal performance regardless of the reduction in CH_4 production. However, results obtained in Chapter 3 confirmed that circulating NO_3 is recycled to the digestive tract via saliva as is urea, providing a continuous supply of fermentable N for microbial growth. This recycling from the GIT into the blood would be highly beneficial in ruminant production in tropical areas. Dietary NO_3 would provide additional N and also reduce enteric CH_4 production in high fibre content diets that have been shown to have the highest CH_4 production per unit of feed eaten (Hristov et al., 2013).

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Journal-Article Format for PhD Theses at the University of New England

General discussion

Statement of originality

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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Name of Candidate: Maria Laura Villar

Name/title of Principal Supervisor: Prof. Roger Hegarty

_____ 12 April 2019

Candidate Date

_____ 12 April 2019

Principal Supervisor Date

Chapter 8. Appendix

8.1. Short communication and oral presentation presented at the Australian Society of Animal Production (ASAP) 2016 conference

Nitrate and canola oil are synergistic in reducing methanogenesis in cattle

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Summary

The effects of canola oil and nitrate (NO_3), added to diets alone or in combination, on methane (CH_4) emissions and rumen volatile fatty acid (VFA) concentrations were evaluated. The experiment was designed as a 4x4 Latin square using 4 cannulated steers over 4 x 15-day experimental periods. Dietary treatments were: control (CON: 40% lucerne chaff and 60% barley grain), OIL (CON + 5% canola oil), NO_3 (CON + 2% nitrate) and NO_3+OIL (CON + 5% canola oil plus 2% nitrate supplied as calcium nitrate). Total VFA concentration and acetate proportion did not differ ($P > 0.05$) between diets, but NO_3 increased the acetate:propionate ratio while OIL reduced it ($P < 0.01$). Compared with CON, NO_3+OIL reduced ($P < 0.01$) methane yield (g CH_4/kg DMI) by 29%. Methane-mitigating effects of nitrate and oil were more than additive and CH_4 emissions were reduced without compromising feed intake or VFA concentration.

Introduction

Enteric methane is produced by ruminants during the microbial fermentation of feed and is an important source of greenhouse gas emitted from the livestock sector (Gerber *et al*., 2013). Strategies that reduce enteric CH_4 emissions are required to minimize agricultural

greenhouse gas emissions. Nitrate and lipids have been evaluated and recognized as effective dietary additives to reduce methane emissions from ruminant livestock (Beauchemin and McGinn, 2006; Guyader *et al*., 2015). The objective of this study was to evaluate the effect of nitrate and canola oil, fed alone or in combination, on rumen fermentation and methane emission from beef cattle.

Materials and Methods

The experiment was conducted between October 2015 and February 2016. Animals were handled in accordance with the University of New England Animal Ethics Committee.

Four mature crossbreed cannulated steers (713 ± 20.5 kg liveweight) were used in a Latin square, with 4 diet treatments offered over 4 periods and each steer fed one of the four dietary treatments in each period.

Animals were housed individually in pens equipped with a feeder and water and were offered 7.5 kg of their experimental diet in 2 equal feeds/d. The basal diet was a blended chaff mixture (40% lucerne chaff; 60% rolled barley grain) fed alone (control; CON) or with inclusion of 2% nitrate (NO_3; provided as 3.14% calcium nitrate, $5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway). The third treatment (OIL) consisted of 5% canola oil inclusion in the chaff and the final treatment (NO_3+OIL) contained 2% nitrate and 5% oil in combination, with all inclusions expressed as g/100g as fed.

Filtered rumen fluid samples were collected for volatile fatty acid (VFA) determination via the rumen cannula after 14 d of diet adaptation. Each 10 ml sample was taken, acidified with 0.3 mL (18M) H_2SO_4 and then frozen at -20°C. Volatile fatty acid concentrations were determined by gas chromatography using a Varian CP-3800 chromatograph.

Methane production (24 h) was measured by open circuit respiration chambers after approximately 13 d (except period 1) and again after 19 d of diet adaption, with data averaged for the 2 measures per animal per period.

Table 1. Dry matter intake (DMI), methane yield, total VFA concentration and VFA molar percentages in rumen fluid of cannulated steers fed: 1) a control diet (CON: 7.5 kg air dry; 40% of lucerne chaff and 60% barley grain; 2) nitrate diet (NO_3: CON + 2% nitrate; 3) oil diet (OIL: CON+5% canola oil) and 4) nitrate plus oil diet (NO_3+OIL: CON with 2% nitrate plus 5% canola oil).

	Treatments				Treatment	
Item	CON	NO_3	OIL	NO_3+OIL	SEM	*P*-value
DMI (kg)	6.8	6.3	6.8	6.3	0.14	0.51
Methane Yield (g/kg DMI)	23.6^{a}	20.1^{b}	22.4^{ab}	16.8^{c}	0.72	<0.01
pH	6.2^{a}	6.3^{a}	6.0^{b}	6.4^{a}	0.03	<0.01
Total VFA (mmol/l)	104	99.5	106.5	100.1	1.61	0.30
VFA (molar %)						
Acetate	62.5	67.5	59.1	64.1	0.42	0.45
Propionate	16.6^{a}	15.2^{ac}	19.6^{b}	15.8^{ac}	0.46	<0.01
Butyrate	17.1^{a}	13.4^{b}	17.0^{a}	16.3^{ac}	0.39	<0.01
Acetate:Propionate ratio	3.8^{a}	4.4^{b}	3.0^{c}	4.0^{a}	0.09	<0.01

$^{a-c}$ Within a row, means without a common superscript letter differ, $P < 0.05$

Results and Discussion

Although NO_3 and NO_3+OIL diets were eaten more slowly (data not shown), there was no difference in daily DMI between treatments (P = 0.51; Table 1). This was probably because feed availability was restricted in this study in contrast to *ad libitum* feeding situations in which a suppressive effect of nitrate on feed intake has often been observed (Weichenthal *et al.,* 1963).

The lack of effect of treatment on DMI was consistent with there being no treatment difference in total VFA concentration, but in all treatments the butyrate percentage was high (13-17 mol%). The OIL treatment caused a significantly lower rumen pH and this was associated with a higher propionate percentage. While microbial reduction of dietary nitrate utilises H_2 and frequently stimulates acetate proportion (Nolan *et al.,* 2010), there was no significant effect of NO_3 (alone or with oil) on the molar percentage of acetate in total rumen VFA. However, the acetate:propionate ratio was higher than the control when NO_3 was fed alone but lower than in the control when oil was fed alone ($P<0.01$). In combination NO_3 and oil did not have a net effect on acetate:propionate ratio.

These effects on DMI, total VFA concentration and molar proportions of individual acids do not completely explain the differential effects of treatment ($P<0.01$) on methane yield (MY: g CH_4/kg DMI). Stoichiometrically, 1 mole of nitrate can be expected to reduce methanogenesis by 1 mol. The inclusion of 20 g of NO_3/kg feed should therefore have reduced methanogenesis by 0.323 mol or 5.17 g/kg intake in this study, whereas the observed mitigation was 3.54 g or 68% of that expected. Similarly, dietary oil has been shown to reduce MY by approximately 1.02 g CH_4/kg DMI per 1% oil added (Grainger and Beauchemin 2011), so the 1.25 g CH_4/kg reduction in MY with oil alone is less than that predicted from including 5% canola oil.

In combination however, NO_3 and oil caused a larger reduction in MY (6.82 g/kg DMI) than the sum of their individual contributions without compromising DMI or total VFA concentration. These findings show that combination of NO_3 and dietary oils as simple feed additives offers a promising means of reducing ruminant enteric emissions. Similar results of additive effects between nitrate and oil were reported by Guyader *et al.* (2015) with nitrate and linseed oil.

Acknowledgements

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8.2. Oral presentation at UNE Postgraduate Conference 2017

Nitrate and canola oil are synergistic in reducing methanogenesis in cattle.

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Enteric methane (CH_4) is produced by ruminants during the microbial fermentation of feed and is an important source of greenhouse gas emitted from the livestock sector. Strategies that reduce enteric CH_4 emissions are required to minimize agricultural greenhouse gas emissions. Because nitrate (NO_3) and lipids alone have been evaluated and recognized as effective dietary additives to reduce methanogenesis in ruminants, we hypothesised that the combination of NO_3 and canola oil would reduce methane emissions in beef cattle. The objective of this study was to evaluate the effect of canola oil and NO_3, added to diets alone or in combination, on CH_4 emissions and rumen fermentation. The experiment was designed as a 4x4 Latin square using 4 cannulated steers over 4 x 15-day experimental periods. Dietary treatments were: control (CON: 40% lucerne chaff and 60% barley grain), OIL (CON + 5% canola oil), NO_3 (CON + 2% NO_3 supplied as calcium salts) and NO_3+OIL (CON + 5% canola oil + 2% NO_3). CH_4 production was measured by open circuit respiration chambers. Daily intake and total volatile fatty acids concentration did not differ ($P > 0.05$) between diets. Rumen ammonia concentration (mg NH_3-N/L) was higher ($P < 0.05$) in NO_3+OIL diet. Compared with CON, NO_3+OIL in combination reduced CH_4 yield (g CH_4/kg DMI) by 29% ($P < 0.01$). Methane mitigating effects of NO_3 and oil are more than additive and CH_4 emissions were reduced without compromising feed intake or rumen fermentation.

8.3. Oral presentation and abstract presented at the Recent Advances in Animal Nutrition (RANN) 2017 conference

Enteric methane reduction in sheep consuming low quality chaff with the addition of nitrate or cysteamine hydrochloride.

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Nitrate (NO_3) is an effective dietary additive to reduce enteric methane (CH_4) emissions from ruminants (Hristov et al., 2013; Lee and Beauchemin, 2014). Rumen microorganisms use hydrogen to reduce NO_3 and decrease H_2 availability for CH_4 production. Furthermore, NO_3 can replace urea (Ur) as a non-protein nitrogen (NPN) source for microbial growth (Leng, 2008) to improve productivity in ruminants consuming protein-deficient diets (Nguyen et al., 2016). A challenge to NO_3 feeding is nitrite (NO_2) toxicity and options to manage the rumen and prevent NO_2 toxicity are being studied (El-Zaiat et al., 2014; Nolan et al., 2016; de Raphélis-Soissan et al., 2017). Like NO_3, cysteamine hydrochloride (CSH) has shown efficacy as a feed additive with potential to reduce enteric CH_4 emissions while improving animal performance (Barnett and Hegarty, 2014; Sun et al., 2017). However, little information about the effectiveness of NO_3 and CSH in low quality diets is available. This study aimed to evaluate the role of NO_3 and CSH as feed additives to reduce enteric emissions while improving productivity in sheep fed low quality chaff.

A complete randomized study using 24 Merino sheep (27.5 ± 0.77 kg LW) was conducted at the University of New England over 35 d. Sheep were assigned to one of four dietary treatments, being wheaten chaff mixture (600 g wheaten chaff and 200 g wheat grain) fed alone (control, CON) or with the inclusion of 2 % NO_3 (provided as 3.14% calcium NO_3), or with inclusion of Ur, iso-nitrogenous to the NO_3 diet or with cysteamine hydrochloride (CSH) at 80 mg CSH/kg BW. A diet adaptation period was included to prevent NO_2 toxicity in NO_3-fed sheep. Feed offered and refused were weighed daily to estimate dry matter intake

(DMI). Filtered rumen fluid samples were collected 3.5 h after morning feeding on Day 35 using esophageal intubation for volatile fatty acids (VFA) analysis, rumen ammonia (NH_3-N) concentration and protozoal enumeration. Animals were offered 800 g of diet and fed once a day. Open circuit respiration chambers (n=6) were used to estimate CH_4 production (DMP) over a 22 h period.

Liveweights of sheep were generally stable throughout the experiment but NO_3-supplementation tended to improve average DMI ($P = 0.10$) and daily gain (ADG, $P = 0.08$; Table 1). Both NO_3 and Ur supplementation increased rumen NH_3-N ($P < 0.01$), confirming the role of these two additives as NPN sources for microbial growth. The rumen protozoa population was reduced ($P < 0.05$) by the addition of NO_3 and by CSH, compared to CON. No differences were observed for rumen VFA concentration between diets but NO_3 increased acetate percentage ($P < 0.05$) and tended ($P = 0.05$) to reduce butyrate percentage compared to Ur. There was a tendency ($P = 0.08$) for NO_3 to reduce enteric DMP and CH_4 yield (MY; g CH_4/kg DMI) was significantly reduced ($P < 0.01$) by NO_3. CH_4 production of CSH supplemented sheep did not differ from CON sheep. These findings confirm the role of NO_3 as a simple feed additive to reduce ruminant enteric emissions, reduce rumen protozoal populations and improve productivity of sheep in low quality diets.

Table 1. Performance, rumen fermentation and methane production by sheep fed low quality chaff without (CON) or with nitrate (NO_3), urea (Ur) or cysteamine (CSH) supplementation.

Parameter		CON	NO_3	Ur	CSH	SEM	*P*-value
Initial liveweight	(kg)	25.8	25.3	25	26.1	0.32	0.23
Final liveweight	(kg)	25.2^{a}	26.0^{b}	23.9^{a}	25.3^{a}	0.34	<0.05
ADG	(g/d)	-23^{a}	18^{b}	-28^{a}	-21^{a}	8.09	<0.10
DMI	(g DM/d)	430^{a}	508^{b}	450^{ab}	492^{ab}	18.00	0.10
Rumen NH_3-N	(mg/L)	37^{b}	136^{a}	137^{a}	64^{b}	0.85	<0.01
Rumen pH		6.7^{a}	6.9^{b}	6.7^{a}	6.7^{a}	0.04	0.05
Total VFA	(mM/L)	58.1	50.7	60.2	59.1	2.45	0.45
Acetate (Ac)	(molar %)	70.7^{ab}	75^{a}	68.4^{b}	70.8^{ab}	1.04	<0.05
Propionate (Pr)	(molar %)	22.1	20.8	24.7	22.7	0.92	0.57
Butyrate	(molar %)	7.2^{a}	4.2^{b}	6.9^{a}	6.4^{a}	0.42	<0.10
Ac:Pr		3.3	3.7	3.0	3.1	0.16	0.44
Total protozoa	($x10^5$/mL)	11.8^{a}	6.1^{b}	9.0^{ab}	5.5^{b}	0.91	<0.05
DMP	(g CH_4/d)	10.3^{a}	7.7^{b}	8.5^{a}	10.9^{a}	0.64	<0.10
MY	(g CH_4/kg DMI)	20.6^{a}	14.8^{b}	19.3^{a}	21.2^{a}	0.06	<0.01

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Enteric methane reduction in sheep consuming low quality chaff with the addition of nitrate or cysteamine hydrochloride

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Background

Nitrate (NO_3) is an effective dietary additive to reduce enteric methane (CH_4) emissions. Furthermore, NO_3 can replace urea as a non-protein nitrogen (NPN) source for microbial growth to improve productivity in ruminants consuming protein-deficient diets. Like NO_3, cysteamine hydrochloride (CSH) has shown efficacy in reducing CH_4 emissions while improving animal performance.

Objective: to evaluate the role of NO_3 and CSH as feed additives to reduce enteric emissions while improving productivity in sheep fed low quality chaff.

Methods

- A complete randomized study with 24 Merino sheep (27.5 ± 0.77 kg live weight) was conducted over 35 days.
- Four dietary treatments were evaluated: wheaten chaff (WC, 600 g and 200 g wheat grain) fed alone (control, CON) or with the inclusion of 2 % NO_3 (3.14% calcium NO_3), or with inclusion of urea (iso-nitrogenous with the NO_3 diet) or CSH (80 mg CSH/kg live weight).
- Animals were offered 800 g of diet and fed once a day.

- A diet adaptation period was included to prevent nitrite toxicity in sheep fed NO_3.
- Open circuit respiration chambers (n=6) were used to estimate CH_4 production (DMP) over a 22 h period.

Results

- NO_3 supplementation tended to improve dry matter intake (DMI) and increased final live weight of sheep.
- Both NO_3 and urea supplementation improved NH_3-N concentration, confirming the roles as NPN source.
- Rumen protozoa population was reduced by NO_3 and CSH compared to CON diet.
- NO_3 significantly reduced methane yield (MY) and tended to reduce DMP

Table 1. Performance, rumen fermentation and methane production of sheep fed low quality chaff without (CON) or with nitrate (NO_3), urea or cysteamine (CSH) supplementation.

Parameter		CON	NO_3	Urea	CSH	SEM	P-value
Initial live weight	(kg)	25.8	25.3	25	26.1	0.32	0.23
Final live weight	(kg)	25.2^{a}	26^{b}	23.9^{a}	25.3^{a}	0.34	< 0.05
DM Intake	(g DM/d)	430^{a}	508^{b}	450^{ab}	492^{ab}	18	0.10
Rumen NH_3-N	(mg/L)	37^{b}	136^{a}	137^{a}	64^{b}	0.85	< 0.01
Rumen pH		6.7^{a}	6.9^{b}	6.7^{a}	6.7^{a}	0.04	0.05
Total VFA	(mM/L)	58.1	50.7	60.2	59.1	2.45	0.45
Acetate (Ac)	(molar %)	70.7^{ab}	75^{a}	68.4^{b}	70.8^{ab}	1.04	< 0.05
Propionate (Pr)	(molar %)	22.1	20.8	24.7	22.7	0.92	0.57
Butyrate	(molar %)	7.2^{a}	4.2^{b}	6.9^{a}	6.4^{a}	0.42	< 0.10
Ac:Pr		3.3	3.7	3	3.1	0.16	0.44
Total protozoa	(10^5/mL)	11.8^{a}	6.1^{b}	9^{ab}	5.5^{b}	0.91	< 0.05
DMP	(g CH_4/d)	10.3^{a}	7.7^{b}	8.5^{a}	10.9^{a}	0.64	< 0.10
MY	(g CH_4/kg DMI)	20.6^{a}	14.8^{b}	19.3^{a}	21.2^{a}	0.06	< 0.01

Row means without common superscripts differ significantly

Conclusions

These findings confirm the role of NO_3 as a simple feed additive for reducing ruminant enteric emissions and rumen protozoal populations and for improving the productivity of sheep fed low quality diets.

8.4. Short communication and oral presentation presented at the Australian Society of Animal Production (ASAP) 2018 conference

Supplementing nitrate as a non-protein nitrogen source for sheep consuming a protein-deficient chaff increases salivary and plasma levels of nitrate or nitrite without risking nitrite toxicity.

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Nitrate is an effective additive to reduce enteric methane emissions and can replace urea as a non-protein nitrogen (NPN) source for ruminants (Nolan *et al.* 2010). A completely randomized study including 24 Merino sheep (27.5 ± 0.77 kg) was conducted over 35 d to investigate the effect of nitrate as NPN source, on plasma or saliva nitrate or urea profiles and blood methaemoglobin (MetHb) levels. Sheep were assigned to one of 3 dietary treatments consisting of 600 g of wheaten chaff (crude protein, CP: 2%; metabolisable energy, ME: 4.9 MJ/kg DM) plus 200 g wheat grain fed alone (control, Con) or with 2 % calcium nitrate (NO_3) or urea (Ur) iso-nitrogenously to the NO_3 diet. An adaptation period was included to prevent nitrite (NO_2) toxicity in NO_3-fed sheep. Animals were fed once daily at 09.30 hours. Blood MetHb concentration was measured once weekly 1.5 h after feeding (AF) throughout the experiment. Blood plasma and saliva samples were collected pre-feeding (PF), 1.5 h and 8 h AF on week 7. Saliva (5 mL) was collected by aspiration with a vacuum pump. Nitrate and NO_2 concentrations were determined in plasma (P-NO_3 or P-NO_2) and saliva (S-NO_3) as described by de Raphélis-Soissan *et al.* (2017). Urea-N was

analysed in plasma (P-urea) and saliva (S-urea) by spectrophotometric analysis (Dade Behring Dimension Xpand Plus, USA).

Averaged blood MetHb concentrations 1.5 h AF in NO_3-fed sheep were 6.74%, below the concentration indicative of NO_2 toxicity (Brunning-Fann and Kaneene 1993). The lowest and highest recorded values were 1.7% and 22.7% of total haemoglobin as MetHb.

In NO_3 treatment, P-NO_3 and P-NO_2 concentrations were greater at 1.5 h AF than for PF and 8 h AF (Table 1; $P<0.05$). Overall, S-NO_3 was higher than P-NO_3 in all dietary treatments and at all sampling times, suggesting that NO_3 was concentrated and recycled via saliva (Leng 2008; Benu 2016); except for PF levels in NO_3-fed sheep in which P-NO_3 was greater than in S-NO_3 ($P=0.03$). However, P-NO_2 was greater at 1.5 h AF ($P=0.02$) but had returned to PF levels by 8 h AF ($P=0.18$) and no visible signs of methaemoglobinaemia were exhibited by sheep. There was a large variation in P-NO_2 between sheep fed NO_3 diet (min=0.4 µM and max=77.9 µM).

Urea concentrations PF and 1.5 h AF were greater in plasma than in saliva ($P<0.001$) whereas no differences were observed 8 h AF for all dietary treatments ($P>0.14$). Across sampling time, supplementing sheep with NO_3 or Ur increased P-urea and S-urea concentrations compared to Con-fed sheep ($P<0.03$), illustrating both supplements would increase salivary N supply for microbial protein synthesis.

We conclude that the addition of 20 g NO_3/kg DM to a protein-deficient diet in ruminants provided advantage through additional N without exposing animals to risk of NO_2 toxicity, even if animals were fed once daily. Our results demonstrate that NO_3 absorption from the rumen into the blood stream increased P-NO_3 and P-NO_2 concentrations and that NO_3 was concentrated in and would be recycled to the rumen via saliva.

Table 1. Nitrate, nitrite or urea concentrations in plasma (P) and saliva (S) measured pre-feeding (PF), 1.5 h or 8 h after feeding in sheep fed a protein-deficient diet supplemented with nitrate (NO_3) or urea (Ur).

Diet	Con			NO_3			Ur				Significance			
Time (h)	PF	1.5	8	PF	1.5	8	PF	1.5	8	SEM	Diet	Time	Site*	DxT
Site-parameter														
P-NO_3 (µM)	4.19	3.82	4.28	25.5	51.8	21.2	3.82	3.76	4.12	2.60	<0.01	<0.01	<0.01	<0.01
P-NO_2 (µM)	1.78	1.68	1.20	4.63	22.9	7.41	2.91	2.42	1.86	0.29	<0.01	<0.01	-	0.01
P-urea (µM)	2063	2081	725	3206	3763	2617	3420	3795	2723	154	<0.01	<0.01	<0.01	<0.01
S-NO_3 (µM)	7.26	12.2	9.97	10.6	165.4	61.1	7.58	8.90	10.3	6.46	<0.01	<0.01	<0.01	0.07
S-urea (µM)	1439	1285	596	1835	2270	1614	2302	3035	1810	111	<0.01	<0.01	<0.01	<0.01

* Site: plasma (P) or saliva (S)

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8.5. Short communication and poster presented at the Australian Society of Animal Production (ASAP) 2018 conference

The effect of protein synthesis inhibitors on gas production by rumen fluid from faunated and defaunated sheep

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Measurement of nitrate (NO_3) and nitrite (NO_2)-reductase activity by rumen microbiota typically relies on quantification of appearance or disappearance of nitrite alone (McNally *et al*. 1997). However, it is hypothesized that production of ammonia (NH_3) could serve as a measure of nitrite reductase activity, removing the requirement for high nitrite concentrations in the assay that are toxic to microbial populations. Measuring NH_3

production would require that incorporation of NH_3 by microbial growth be inhibited, as is performed in protein degradability studies (Broderick 1987) without adversely affecting fermentation. A study was conducted using gas production by rumen fluid from faunated or defaunated (protozoa-free) sheep to assess the effect of microbial protein synthesis inhibitors on ruminal fermentation, prior to including these inhibitors in further assays for nitrate- and nitrite-reductase activity determinations.

In vitro incubations were conducted using whole rumen fluid samples (70 mL) collected from two crossbred female sheep (defaunated; n = 1; 87.6 kg) and faunated (n = 1; 71.8 kg) by oesophageal intubation three hours post-feeding. Both sheep had been acclimated to dietary NO_3 (2 % of diet DM) provided as calcium nitrate ($5Ca(NO_3)_2$. $NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway), sprayed on blended lucerne-oaten chaff.

Each 70 mL rumen fluid sample from the faunated (F) and defaunated (D) sheep was subsampled (2 x 30 mL) into 50 mL plastic syringes containing ground samples of NO_3^- supplemented dietary chaff (301 ± 6.6 mg DM chaff/syringe) and fitted with three-way luer-lock taps. Protein inhibitor (+I)was added (3.9 mg hydrazine sulphate ($H_6N_2O_4S$) and 0.9 mg chloramphenicol, $C_{11}H_{12}Cl_2N_2O_5$) to one syringe tube from each sheep, with no inhibitor added to the remaining tube (-I). Incubations were run for 4h with headspace gas quantified after 1, 2, 3 and 4 h.

A repeated measures analysis over time indicated that there was no effect of protozoa on gas production ($P>0.05$) and the effect of inhibitor was dependent upon time ($P<0.01$). Although protein synthesis inhibitors increasingly repressed gas production over time, the suppression of gas production after 1h was minimal (Figure 1). As incubations for nitrate reductase assays are typically conducted for 15 min only, it was determined that inclusion of protein synthesis inhibitors would not significantly affect short-term fermentation, so could be used to enable estimation of nitrite reduction based upon the accumulation of ammonia within the incubation.

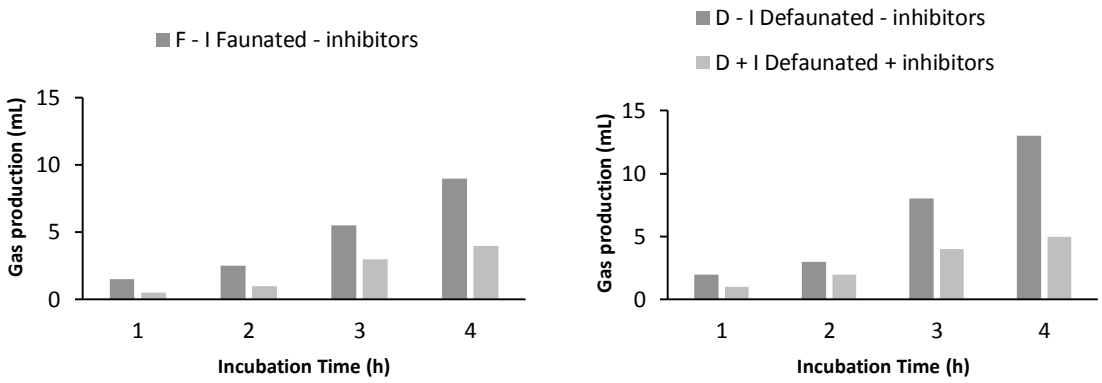

Figure 1. Gas production over time by whole rumen fluid collected from faunated and defaunated sheep acclimated to 2 % dietary NO_3^- in DM. Incubations were conducted at 39˚C with or without protein synthesis inhibitors (3.9 mg hydrazine sulphate + 0.9 mg chloramphenicol).

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2637 The effect of protein synthesis inhibitors on gas production by rumen fluid from faunated and defaunated sheep

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Introduction

Measurement of nitrate (NO_3^-) and nitrite (NO_2^-)-reductase activity by rumen microbiota typically relies on quantification of appearance or disappearance of NO_2^- alone, however it is hypothesised that production of ammonia (NH_3) could serve as a measure of NO_2^- reductase activity, removing the requirement for high NO_2^- concentrations in the assay that are toxic to microbial populations. Measuring NH_3 production would require that incorporation of NH_3 by microbial growth be inhibited as is performed in protein degradability studies, without adversely affecting fermentation. A study was conducted using gas production by rumen fluid from faunated or defaunated (protozoa-free) sheep to assess the effect of microbial protein synthesis inhibitors on ruminal fermentation, prior to including these inhibitors in further assays for NO_3^- - and NO_2^- -reductase activity determinations.

Materials and Methods

In vitro incubations were conducted using whole rumen fluid samples (70 mL) collected from one faunated (71.8 kg) and one defaunated (87.6 kg) crossbred ewes by oesophageal intubation three hours post-feeding. Both sheep had been acclimated to dietary NO_3^- (2 % of diet DM) provided as calcium nitrate ($5Ca(NO_3)_2.NH_4NO_3.10H_2O$), Bolifor CNF, Yara, Oslo, Norway, sprayed on blended chaff.

Each 70 mL rumen fluid sample from the faunated (F) and defaunated (D) sheep was subsampled (2 x 30 mL) into 50 mL plastic syringes containing ground samples of NO_3^- supplemented dietary chaff (301 ± 6.6 mg DM chaff/syringe). Protein inhibitor (3.9 mg hydrazine sulphate ($H_6N_2O_4S$) and 0.9 mg chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$;+I)) was added to one syringe tube from each sheep, with no inhibitor added to the remaining tube (-I). Incubations were run for 4h in a shaking water bath with headspace gas quantified after 1, 2, 3 and 4 h.

Key Learnings

- A repeated measures analysis over time identified there was no effect of protozoa on gas production ($P>0.05$) and the effect of inhibitor was dependent upon time ($P<0.01$)
- Although protein synthesis inhibitors increasingly repressed gas production over time, the suppression of gas production after 1h was minimal (Figure 1)
- As incubations for NO_3^- reductase assays are typically conducted for 15 min only, it was determined that inclusion of protein synthesis inhibitors would not significantly affect short-term fermentation, so could be used to enable estimation of NO_2^- reduction based upon the accumulation of NH_3 within the incubation

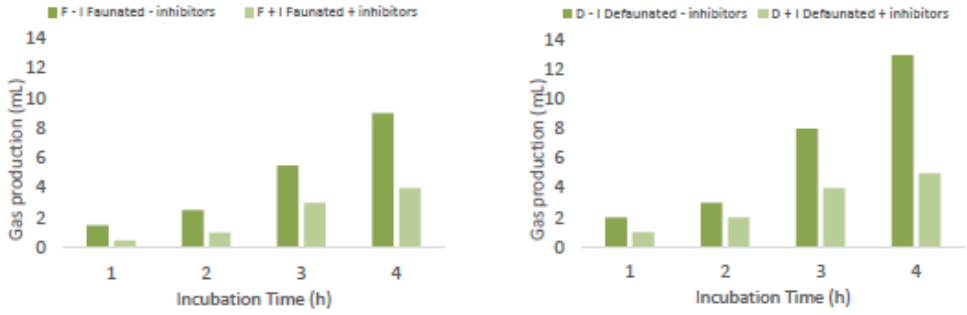

Figure 1. Gas production over time in whole rumen fluid collected from faunated and defaunated sheep acclimated to 2 % dietary NO_3- in DM. Incubations were conducted at 39°C with or without protein synthesis inhibitors (3.9 mg hydrazine sulphate + 0.9 mg chloramphenicol).

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8.6. Rumen and reticulum samplers

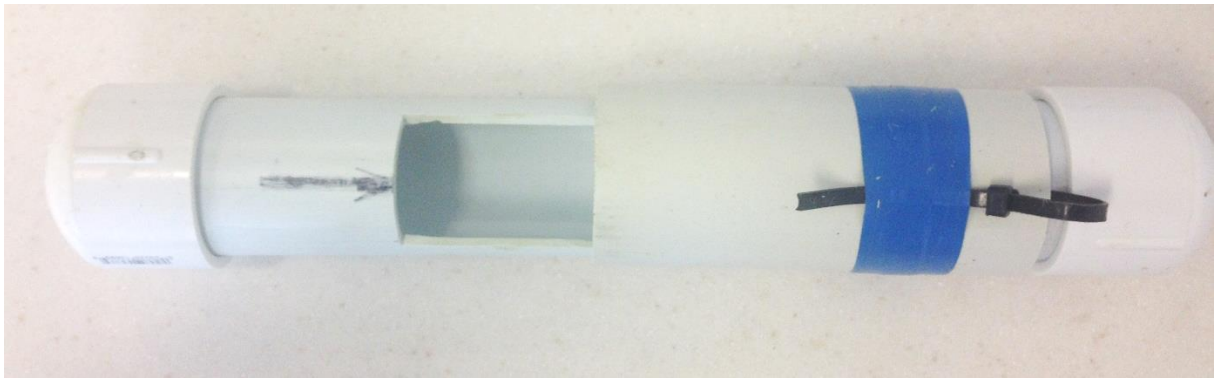

Figure 8.1 Rumen sampler

Figure 8.2 Reticulum sampler

8.7. Frozen pellets of oaten hay mordanted with chromium

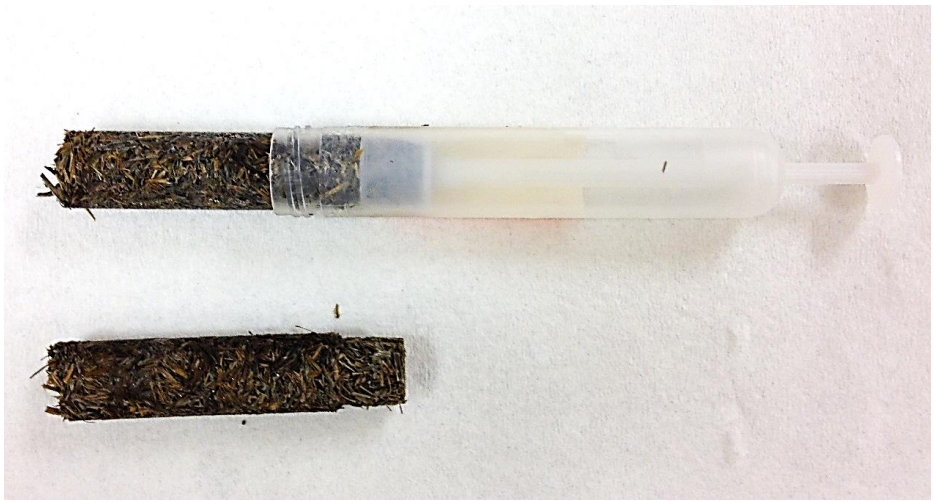

Figure 8.3 Frozen pellets containing neutral detergent fibre (NDF) from oaten hay mordanted with chromium (2.5 g of mordanted NDF, 149.5 mg Cr/pellet)

Journal-Article Format for PhD Theses at the University of New England

Appendix

Statement of originality

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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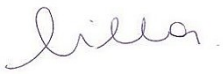

_____ 12 April 2019

Candidate Date

_____ 12 April 2019

Principal Supervisor Date