Nutritional strategies to reduce nitrogen excretion in beef cattle

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B.Agr.Sc.

(Animal and Crop Production)

June 2024

A dissertation submitted to the National University of Ireland, Dublin (University College Dublin) in fulfilment of the requirements for the Degree of Doctor of Philosophy

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Declaration

I hereby declare that the work reported herein is my own and that this thesis has not been previously submitted as an exercise for a degree at the National University of Ireland, or any other University.

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Stuart Kirwan, June 2024

Glossary of terms

AA	Amino acid
ADF	Acid detergent fibre
ADG	Average daily gain
ALE	*Alaria esculenta*
AREC	Animal Research Ethics Committee
ASC	*Ascophyllum nodosum*
ATP	Adenosine triphosphate
BO	Blackseed oil
BW	Body weight
CO	Control
C1	Chitosan molecular weight <5,000
C2	Chitosan molecular weight 10,000
C3	Chitosan molecular weight 20,000
C4	Chitosan molecular weight 150,000
$C0_2$	Carbon dioxide
CH_4	Methane
CHI	Chitosan
CP	Crude protein
CSO	Central Statistics Office
CLRTAP	Convention on Long Transboundary Air Pollution
cm	Centimetre
d	Day
DAFM	Department of Agriculture, Food and the Marine
Da	Daltons
DM	Dry matter
DMI	Dry matter intake
EEA	European Economic Area
ED	Effective degradability
EO	Essential oils
EPA	Environmental Protection Agency
ETS	Emissions trading scheme
EU	European Union
eq	Equivalent
FAO	Food and Agriculture Organisation
g	Gram
g	Gravity
GC	Gas chromatography
GHG	Green house gas
GS	Grass silage
g/day	Gram per day
H	Hydrogen
H_2	Hydrogen gas
H	Hour
HAP	Hyper ammonia producing bacteria
HIM	*Himanthalia elongate*
HP	High protein
HPRA	Health Products Regulatory Authority
H_20	Water

id	Internal diameter
kDa	Kilodaltons
Kg	Kilogram
Km	Kilometre
KO%	Kill out %
kt	Kiloton
L	Litre
LP	Low protein
M	Metre
ME	Metabolisable energy
Mg	Milligram
Ml	Millilitres
Mm	Millimeter
MM	Maize meal
mmol	Millimoll
mt	Megaton
MS	Maize silage
N	Nitrogen
N	Number
NaCl	Sodium chloride
$NaSO_3$	Sodium sulphite
NDS	Neutral detergent solution
NH_3	Ammonia
NH_3^+	Protonated amino groups
N_2O	Nitrous oxide
NDF	Neutral detergent fibre
NH_4	Ammonium
NO	Nitric oxide
NS	Non-significant
NUE	Nitrogen use efficiency
O_2	Oxygen
OMD	Organic matter digestibility
OM	Organic matter
pKa	Acid dissociation constant
pH	Power of hydrogen
$PM_{2.5}$	Fine particulate matter
PT	Phlorotannins
R^2	Root squared
RB	Rolled barley
RDP	Rapidly degradable protein
RUSITEC	Rumen simulation technique
RUP	Rumen undegraded protein
SO_2	Sulphur dioxide
SD	Standard deviation
SEM	Standard error of the mean
SH	Soya hulls
SI	Small intestine
T	Tonne
TCA	Trichloroacetic acid
TMR	Total mixed ration

UCD	University College Dublin
UK	United Kingdom
V	Volume
VFA	Volatile fatty acids
yr	Year
W	Weight
WSC	Water soluble carbohydrates
° C	Degrees celsius
µl	Micro litres
µm	Micro metre
2H	Metabolic hydrogen

Acknowledgements

Firstly, I would like to give a sincere thanks to my supervisors, Prof. Tommy Boland, and Prof. Karina Pierce, for providing me with the opportunity to undertake this PhD. For all your valuable advice, guidance, and expertise you both afforded me when designing and analysing the experiments. To the countless hours correcting, and above all for your patience, and encouragement throughout the course of this thesis. Working with you both, not only have I expanded my knowledge in ruminant nutrition but become more proficient in how to interpret science more effectively. I would also like to thank Dr. Alan Kelly for his help and guidance with the statistical analysis.

I would also like to acknowledge the Department of Agriculture, Food, and Marine for their financial support in facilitating this research and the Lyons Management Committee for providing the facilities at Lyons to carry out my research work. A special thanks to Stephen Lott for acquiring the quietest animals for my trial work, but for your unending positivity, and humour making the whole experience in the yard so much easier and more enjoyable. Thanks to the other farm staff Michael, Ann-Marie Ian, Niall, Michael, Noel and Joe for all your help also.

Prof. Vivian Gath, for your expertise and surgical skills that were essential to facilitate the collection of samples necessary to complete this thesis. There was never a dull moment when you were around!

To the technical staff at Lyons: Mr. Pat Duffy, for your expertise and advise in the preoperative and postoperative animal care, and your tutelage in the numerous sampling procedures that were ascertained during the course of this PhD, but also during my time at Lyons. Mr. Pat Quinn, firstly, as special thanks for keeping us supplied with tea bags

and milk for our well-earned tea breaks, but also for your help during the collection periods in the metabolism house. In addition, a special thanks to Mr. Michael Mc Donald for your help with the rumen sampling in the metabolism during the second animal study, staying on late into the night, allowing me to catch up on some long overdue sleep. Special thanks to Ms Mary Wade for ensuring the surgical instruments were clean and available when required. To Eugene Brennan, for keeping the Calan Data Ranger alive, to keep my animals fed during my experiments.

Thanks to Ms Bernie Flynn and Dr. Gaurav Rajauria for your help and guidance in the lab, and to Paddy and Denise for analysing samples in Belfield.

Thanks to all the students who passed through Lyons during my time there: Áine Frank, Fiona, Enda, Eamon, John, Mark, Sally, Louise, Connie, Billy, Zoe, Noel, Meike, Sarah, Orla, Cathal, Ruth, Shane, Marco, Michelle, Gitta, Ciaran, Elaine, MJ, Conor, Ciaran, Kate, Jonathan, Fionnuala, and Shona. Thanks to all of you who helped with sampling in the metabolism house and in the RUSITEC lab, your friendship, banter over the years and for making the whole experience so much more enjoyable. An additional thanks to work placement students: Ian Castles, Ania, Paulina, and Elodie but especially Eleonora Serra for their help with my trial work.

An additional thank you to Zoe, Connie and Conor for their friendship, support, proof reading and helping to put this thesis together.

Finally, to my family, Fran, Mel, Michael, Michelle, Ross, Lisa, and Eimhear, I want to thank you all for the constant support and encouragement throughout my thesis, especially during the final stages.

Abstract

Ruminant production systems have a negative impact on the environment and are a major contributor to atmospheric ammonia (NH_3) emissions. Therefore, there is a need to investigate dietary strategies and novel feed additives for ruminants that reduce nitrogen (N) excretion. The two overall aims of this thesis are: 1) To investigate the effects of low crude protein (CP) diets, different carbohydrate sources and novel feed ingredients on nutrient intake and digestibility, ruminal fermentation, and N excretion in beef heifers. 2) To evaluate novel feed additives on ruminal fermentation parameters *in vitro* using the rumen simulation technique (RUSITEC). To achieve these aims, four experiments were carried out. The first experiment evaluated the effect of supplementing grass silage based diets with differing carbohydrate sources. Offering a carbohydrate source that is rapidly degraded in the rumen, such as, rolled barley did not alter ruminal NH_3 concentrations nor reduce N excretion in beef heifers. The second experiment investigated the effects of chitosan differing in molecular weight, at different inclusion levels on the manipulation of rumen fermentation *in vitro* using RUSITEC. Chitosan inclusion had little impact on rumen fermentation *in vitro*. The third experiment also investigated the effects of chitosan inclusion at two levels of dietary CP on nutrient intake and digestibility, ruminal fermentation, and N excretion in beef heifers. Chitosan inclusion increased ruminal NH_3 concentrations in high CP diets while having no effect on N excretion. Finally, the fourth experiment evaluated the effects of supplementing diets with different CP levels with different brown seaweed species on rumen fermentation *in vitro* using RUSITEC. Inclusion of brown seaweeds reduced NH_3 concentrations in both high CP and low CP diets, but not sufficiently to affect microbial fermentation. The findings of this thesis indicate that dietary strategies (altering CP levels and carbohydrate sources) and inclusion of brown

seaweeds offer potential to mitigate against N excretion in cattle, while acknowledging that further research is required.

Chapter 1

General introduction

1.1 Introduction

The Irish agri-food sector is Ireland's most important indigenous industry. In 2020, the sector accounted for 7.5% of Gross National Income, employing approximately 164,400 people (7.1% of total employment) supporting employment in rural and coastal areas in particular (DAFM, 2021). The sector is export orientated; with 90% of food, produced, exported, leaving the sector vulnerable to global markets (Ryan et al., 2016). Ireland is the fifth largest net exporter of beef in the world, with the Irish beef production industry responsible for 28% of gross output, estimated to be worth €2.2 billion in 2019 (Bord Bia, 2020). However, the outlook for the industry is not positive, as production is expected to decline, influenced by the shrinking of the national beef-cow herd, low profitability, declining demand for beef within the European Union (EU) and competition from the dairy sector (Power, 2020). While numbers in the national beef cowherd continue to decline, any environmental benefits associated with these reductions have been offset by the steady increase in cow numbers in the national dairy herd. As agriculture is responsible for 37.1% of Irelands total greenhouse gas emissions and 99.2% of ammonia (NH_3) emissions, the agricultural industry faces a challenge to produce food in a more sustainable manner (Duffy et al., 2020).

Nitrogen losses into the environment from agricultural livestock systems have the potential to negatively impact air quality causing respiratory disease due to exposure to high concentrations of fine particulate matter ($PM_{2.5}$); nitrate contamination of drinking water; eutrophication of water courses; decreased biodiversity in sensitive ecosystems due to higher concentrations of N; contribute to greenhouse gas emissions with increases in nitrous oxide (N_2O); saturation of forest floors with N; and soil acidification through nitrification and leaching (Cole et al., 2006; Ndegwa et al., 2008;

Hristov et al., 2011). The agricultural sector is responsible for 99.2% and 92.2% of NH_3 and N_2O emissions respectively (EPA, 2021). The livestock sector accounts for 88.6% of the national NH_3 emissions in Ireland, with dairy and non-dairy bovines accounting for 38.2%, and manure management accounting for 46.7% of NH_3 emissions (EPA, 2021).

Ireland is party to the Convention on Long Range Transboundary Air Pollution (CLRTAP) under which specific transboundary air pollutants including NH_3 are controlled (DAFM, 2019). As a member of the EU, Ireland is obliged to implement and reach targets set out in the Gothenburg protocol [NEC Directive (2016/2284/EU)], later amended under the EU Clean Air Package, Directive (2016/2284/EU). Thus, committing emission targets for NH_3 of 1% below 2005 for the period 2021 to 2029 and 5% below 2005 for the period beyond 2030, setting a new target of 107 kt. However, with the anticipation and removal of milk quotas in 2015, NH_3 emissions have been on an upward trend since 2011 because of increased dairy and their dairy-beef offspring (EPA, 2021).

Nitrogen retention in beef cattle is low at 10-20% (Cole and Todd, 2009) compared to dairy cattle 25-34% (Huhtanen and Hristov, 2009), resulting in large amounts of ingested N being excreted in the urine and faces. The loss of N in animal excreta occurs when urea N (mainly in the urine) is hydrolysed by the enzyme urease, which is abundantly available in faeces and soil following deposition from livestock during housing and when animals are grazing, manure storage and land application (Oenema et al., 2007). Urinary N, which is mostly in the form of urea, has a greater impact on NH_3 emissions than faecal N, as its readily volatilised to NH_3 (Lanigan et al., 2008) compared to N in faeces which is in more complex organic forms that are transformed through slower mineralisation processes into reactive compounds (Todd et al., 2015).

In Ireland, grass silage (GS) is the main forage fed during the indoor-winter period with supplementation of concentrates offered mainly to finishing cattle, and to compensate for low quality conserved forages (McGee et al., 2006). Following the ensiling process, the main carbohydrate substrates available for fermentation in the rumen are slowly fermented plant cell walls, while the N substrates in GS are mainly soluble and very quickly available leading to an asynchronous release between energy and N in the rumen (Van Vuuren et al., 1993). Supplementing with different types and amounts of carbohydrates in the diet has been shown to affect N excretion (Castillo et al., 2001). The incorporation of ruminal NH_3 into microbial protein is greater with carbohydrates that are highly fermentable and more rapidly available in the rumen such as starch and sugars, compared to other carbohydrate sources such as cellulose and hemicellulose (Stern and Hoover, 1979; Hasan, 2015). Undigested protein is excreted in the faeces, while digested protein is either converted into animal tissue or milk protein or converted to urea in the liver and excreted in urine by the kidneys (Satter et al., 2002). The relationship between dietary crude protein (CP) or N intake and N excretion in the urine is far stronger (R^2 = 0.74) than faecal N excretion (R^2 = 0.21) and milk N excretion (R^2 = 0.30) (Mulligan et al., 2004) and strategies that reduce CP are generally associated with a reduction in urinary N output (Mulligan et al., 2004; Broderick et al., 2008). Reducing the intake of dietary CP has been shown to reduce urinary N excretion and improve N use efficiency (NUE) (Menezes et al., 2016). Cole et al. (2006) observed no performance difference between finishing cattle offered diets containing 11.5% and 13.0% CP. Furthermore, during the finishing period of 161 d, N excretion decreased 0.1 to 2.9 kg animal^{-1} and the estimated rate of N volatilization decreased by approximately 4.4 kg animal^{-1}. Similarly, feeding CP in excess to the animals requirements resulted in a higher urea output in the urine (Koenig

and Beauchemin, 2013). Therefore, reducing dietary CP intake is an important strategy in reducing urinary N excretion and potential N loss to the environment.

Altering the N excretion pathway from urinary N towards faecal N is more favourable because faecal N is much less prone to environmental losses compared to urinary N (Brinkhaus et al., 2016). Several nutritional strategies have demonstrated the ability to improve rumen fermentation and decrease N excretion, including better-feed formulation to increase animal performance and feed utilization and modifying rumen function using various feed additives. The most promising results to date have been obtained from the use of probiotics, dietary lipids, organic acids, enzymes, and plant secondary compounds (Cobellis et al., 2016b), reducing overall N excretion, and/or shifting the excretion pathway from urinary to faecal, and improving overall NUE (Jouany and Morgavi, 2007).

Many plants synthetize organic compounds as secondary metabolites (Patra and Saxena, 2011). They are not involved in their primary biochemical processes, such as growth, development, or reproduction, but they are important for protection against insect predation and microbial infection (Greathead, 2003). In the past, animal nutritionists considered plant secondary metabolites as anti-nutritional factors due to adverse effects on nutrient utilization (Cobellis et al., 2016a). Recently, many plant extracts have been studied for their antimicrobial activity and ability to modify gut function in both ruminant and non-ruminant animals (Wallace, 2004). Some plant secondary metabolites, such as saponins, tannins and essential oils, have shown potential to improve rumen metabolism, such as decreased methanogenesis and protein degradation in the rumen, increased microbial protein production and protein flow to the duodenum (Wallace, 2004; Muller-Harvey, 2006; Benchaar et al., 2008; Hart et al., 2008; Patra and Saxena, 2009).

Most research to date has focused on the use terrestrial tannins, as their effect on rumen metabolism is more consistent compared to other secondary compounds (Patra and Saxena, 2009). Terrestrial tannins are phenolic secondary compounds in plants, which are widely distributed in forages, legumes, cereals, grains, trees, and shrubs (Patra & Saxena, 2011; Min et al., 2003). They have the ability to form tannin-protein complexes in the rumen (Patra and Saxena, 2011). Furthermore, they inhibit the growth and activities of proteolytic bacteria and have been shown to shift the site of N metabolism from the rumen to the lower digestive tract and large intestine (Min et al., 2003; de Klein and Eckard, 2008), shifting N excretion from urinary to faecal and increasing overall NUE (Misselbrook et al., 2005; Grainger et al., 2009; Hymes-Fecht et al., 2013; Wischer et al., 2014). Seaweeds contain a broad spectrum of nutritional compounds, polysaccharides, polyunsaturated fatty acids, and polyphenols, such as phlorotannins (PT) (Kumari et al., 2010; Tierney et al., 2010; Fitzgerald et al., 2011). Nevertheless, the effect of PT on rumen metabolism is still in its infancy and studies to date have produced conflicting results (Wang et al., 2008; Wang et al., 2009; Belanche et al., 2016a; Vissers et al., 2018). Further research in this area is required to determine the potential effect of PT on N metabolism in ruminants.

Chitosan (*N*-acetyl-D-glucosamine polymer) is a natural biopolymer formed from the deacetylation of chitin (Belanche et al., 2016b). Due to its biodegradability and non-toxic properties, chitosan has received much attention for its diverse applications in medicine and food preservation due to its antimicrobial properties (Cuero, 1999; Shahidi et al., 1999; Jeon et al., 2002). Previous studies demonstrated the positive antimicrobial, anti-oxidative and immunoregulatory effects of supplementing pigs and poultry diets with chitosan, such as enhanced nutrient digestibility and growth performance (Swiatkiewicz et al., 2015; Guan et al., 2019). Its use in ruminant

nutrition has not been extensively investigated, with studies to date reporting conflicting results (Goiri et al., 2010; Araújo et al. 2015; Mingoti et al., 2016; Vendramini et al., 2016; De Paiva et al., 2017; De Valle et al., 2017; Dias et al., 2017). Goiri et al. (2010) demonstrated that chitosan inclusion had the ability to shift rumen fermentation pattern towards a more energy efficient pathway when included *in vitro* while simultaneously reducing ruminal NH_3. Other studies showed that chitosan inclusion could affect ruminal N metabolism in a dose-dependent manner (Araújo et al. 2015; Dias et al., 2017). However, further studies are required to investigate the effects of chitosan inclusion on N metabolism in ruminants offered grass silage diets.

1.2 Thesis objectives and hypothesis:

The two overall objectives of this thesis were to:

1) Investigate the effects of low protein diets, different concentrate carbohydrate sources with different fermentation profiles, and novel feed ingredients on nutrient intake and digestibility, ruminal fermentation, and N excretion in beef heifers offered a grass silage based diet.
2) Evaluate different novel feed ingredients on ruminal fermentation parameters *in vitro* using the rumen simulation technique (RUSITEC).

The hypotheses of this thesis were as follows:

- Offering a carbohydrate that is rapidly degraded within the rumen, will in turn capture more N within the rumen and reduce N excretion.
- Offering chitosan to beef cattle will alter N metabolism within the rumen, increase CP digestibility in low CP diets, and in turn reduce N excretion.

- The inclusion of chitosan, low in molecular weight will inhibit ruminal degradation, and in turn reduce ruminal NH_3 concentrations *in vitro.*
- The inclusion of brown seaweeds will inhibit ruminal protein degradation and reduce ruminal NH_3 concentrations without affecting rumen fermentation in diets differing in CP content *in vitro.*

These hypotheses were investigated through a literature review and four research chapters as follows:

Chapter 2 provides a detailed review of the literature relating N metabolism within the rumen and the effects of dietary additives can have on N excretion.

Chapter 3 evaluates the effect of supplementing grass silage-based diets with concentrate carbohydrate sources with different fermentation profiles, on N metabolism of beef heifers fed to maintenance.

Chapter 4 assesses the effects of chitosan inclusion at different feeding levels and molecular weight, on on ruminal fermentation parameters *in vitro* using the RUSITEC.

Chapter 5 evaluates the effects of chitosan inclusion and dietary CP level on nutrient intake and digestibility, ruminal fermentation, and N excretion in beef heifers offered a grass silage based diet.

Chapter 6 assesses the effects of dietary CP level and brown seaweed source on ruminal fermentation parameters *in vitro* using the RUSITEC.

Chapter 7 provides an overall discussion of the scientific findings and potential future research pathways.

Finally, **Chapter 8** provides an overview of the scientific and conference publications that resulted from the work carried out as part of this thesis.

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Chapter 2

Literature review

2.1 Introduction

The purpose of this chapter is to review the literature available relating to the environmental impact of ammonia (NH_3) emissions from beef production and factors that influence these emissions, nitrogen (N) digestion and metabolism in ruminants and dietary strategies to reduce N excretion from the beef animal.

The literature review presented is divided into two main sections. The purpose of the first section is to give a background to the Irish beef industry, its importance to the Irish economy and the different types of production systems. The contribution of Irish agriculture, in particular the ruminant sector, to greenhouse gas (GHG) and NH_3 emissions to the environment are discussed. The current legislation relating to NH_3 emissions (Gothenburg Agreement/NEC Directive 2016/2284/EU), and nitrous oxide (N_2O) (Kyoto Protocol) are also presented, as are mitigation strategies to reduce these losses from ruminant production systems.

The second section of this review deals with digestion and metabolism of crude protein (CP) in the beef ruminant. Factors that affect the digestion of CP, the influence that protozoa have on N metabolism in the rumen. Urea recycling in the ruminant is discussed along with oscillating the CP level in the diet to improve or enhance urea recycling in beef finishing diets. Finally, the role of dietary additives and the impact they have on reducing N excretion in beef cattle are reviewed.

2.1.1 Irish agricultural industry

Agriculture is an important and fundamental component of Irish economic, social, and cultural history but in recent times, Irish agriculture has undergone substantial changes in how it is carried out (EPA, 2016). The land area of Ireland is 6.9 million hectares, with 4.4 million hectares used for agriculture and of this 81% is devoted to grassland

production (pasture, hay, and grass silage (GS)) with a further 11% classed as rough grazing (CSO, 2021). In 2016, there were 137,100 farms in Ireland, with specialist beef production the most common farming system, 78,300 farms (CSO, 2018). Farms in the Southern and Eastern regions tend to be larger (38.6 hectares) than those in the Border, Midlands, and Western Regions (27.1 hectares) with less than half (47.3%) of all farms located in the Southern and Eastern regions. In economic terms, the cattle rearing sector is the least profitable type of farming in Ireland compared to the dairy sector which has the highest profitability among all sectors (DAFM, 2019).

2.1.2 Value of beef industry to the Irish economy

The Irish beef industry currently accounts for about 56.7% of the combined value of Irish meat and livestock (Bord Bia, 2020). Ireland is the largest net exporter of beef in the EU (within the EU, half of Irish exports shipped to the UK) and sixth largest in the world (Hennessy et al., 2018). Ireland is self-sufficient in beef production, estimated at 650%, producing over 624,000 tonnes in 2019, of which almost 90% is exported, worth approximately €2.2 billion (Bord Bia, 2020).

2.1.3 Beef production systems

Beef farming in Ireland is composed of many different production systems, where cattle are sold off farms for further finishing or else sold direct to slaughter. Beef cattle slaughtered in Ireland originate from two sources: national suckler herd (940,000 sucker cows) and national dairy herd (1.6 million dairy cows) which ensures an annual calf-crop of approximately 1.9 million calves entering beef production (CSO, 2021b). There are 135,037 farms in Ireland with over 74,159 specialising in beef (CSO, 2021a). Since the removal of EU milk quotas in 2015, the Irish dairy herd is undergoing substantial expansion, which has seen an increase in the number of dairy progeny entering the beef supply chain. Beef animals from the dairy herd tend to have

poorer conformation and kill-out percentage compared to their suckler herd counterparts, however from a sustainability perspective dairy beef has a much lower GHG than suckler beef (Burke, 2016).

Beef production in Ireland is dominated by pastoral-based systems, typically fed grass-based diet (Lanigan et al., 2017) and conserved forages which is predominantly GS (0.87 of conserved forage in Ireland) (Mayne and O' Kiely, 2005). Cattle typically spend about 60% of their time at pasture with the remainder spent indoors. In 2019, 90.6% of the NH_3 emissions from agriculture was accounted by animal manures, with manure management accounting for 47.4% while grazing animals responsible for 10.9% (EPA, 2021).

2.2 Emissions from Irish agriculture

Irish agriculture faces a major challenge concerning climate change, in how it deals with GHG emissions and transboundary gases (Duffy et al., 2019). Agriculture in Ireland is the largest contributor to overall GHG, accounting for 37.1% of total emissions (EPA, 2021). These emissions from the agricultural sector are uniquely high compared to other European countries where the average is 10.1% and reflects the importance of agriculture to the Irish economy (O′ Mara et al., 2021), which is predominantly based on the export of ruminant livestock products. The GHG relevant to agriculture are Carbon dioxide (CO_2), N_2O, Methane (CH_4) with emissions dominated by CH_4 and N_2O (McGettigan et al., 2010). Methane is the most significant GHG in agriculture and results from enteric fermentation and manure management, with enteric fermentation accounting for 57.7% (O′ Mara et al., 2021), and N_2O 32.2% of total agricultural emissions (Duffy et al., 2019). Emissions from agriculture

increased in 2015 by 1.6% as a result from increased dairy cattle numbers and urea application (EPA, 2021).

2.2.1 Ammonia emissions

Ammonia is a major pathway for reactive N entering the atmosphere and subsequently being deposited to terrestrial and aquatic ecosystems (Todd et al., 2015). Ammonia impacts on environmental sustainability as it can lead to eutrophication of nearby water courses, react with acid pollutants such as SO_2 and NO_x emissions to produce aerosols (Particulate Matter <2.5 µm diameter $PM_{2.5}$) which leads to significant negative impacts on human health and the environment, lead to increased GHG as deposition can result in indirect N_2O losses. The agricultural sector is responsible for essentially all (99.2%) of NH_3 emissions in Ireland (EPA, 2021). The loss of N in animal excreta occurs when urea N (mainly in the urine) is hydrolysed by the enzyme urease, which is abundantly available in faeces and soil following deposition from livestock during housing, manure storage and land application and when animals are grazing (Oenema et al., 2007). Urinary N - which is mostly in the form of urea - has a greater impact than faecal N, as its readily volatilised to NH_3 (Lanigan et al., 2008) compared to N in faeces which is in more complex organic forms that are transformed through slower mineralisation processes into reactive compounds (Todd et al., 2015). Ammonia is lost during slurry application, animal housing, slurry storage, grazing, inorganic fertilizer application and from crops (Bussink and Oenema, 1998). In 2019, storage of animal manures, the application of organic manures, and with urine and dung deposited by grazing animals accounted for 47.4 %, 32.3%, 10.9% respectively, share of the total NH_3 emissions from agriculture in Ireland (EPA, 2021).

2.2.2 Nitrous Oxide

In 2019, Nitrous Oxide (N_2O) emissions from agriculture accounted for 92.2% of total N_2O emissions (EPA, 2021) largely driven by increases in dairy cow numbers. Nitrous Oxide is a GHG with 298 times the global warming potential of CO_2 (Forster et al., 2007), and a lifetime of 120 years (IPCC, 1996; Lanigan, 2017). Nitrous oxide in the stratosphere reacts with atomic oxygen to form nitric oxide (NO) which induces the destruction of stratospheric ozone (Bliefert, 1994).

Nitrous oxide emissions arise from agricultural soils as a result of microbial nitrification and denitrification of mineral N following the application of inorganic fertilisers and animal manures (Charles et al., 2017). Nitrification is the aerobic oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) arising from the mineralisation of organic N during the decomposition of plant residues and animal manures (Nevision et al., 2016; Charles et al., 2017). Denitrification is facilitated by facultative anaerobic bacteria in the reduction process of NO_3^- to dinitrogen gas (N_2) (Charles et al., 2017), which accounts for a major loss of fixed N from soils and oceans (Nevision et al., 2016). The yield of N_2O from the soil depends on many variables including organic carbon availability, O_2 partial pressure, soil moisture content, soil pH, and temperatures (Davidson and Ewank, 1986; Liu et al., 2011; Giacomini et al., 2006; Signor & Cerri, 2013).

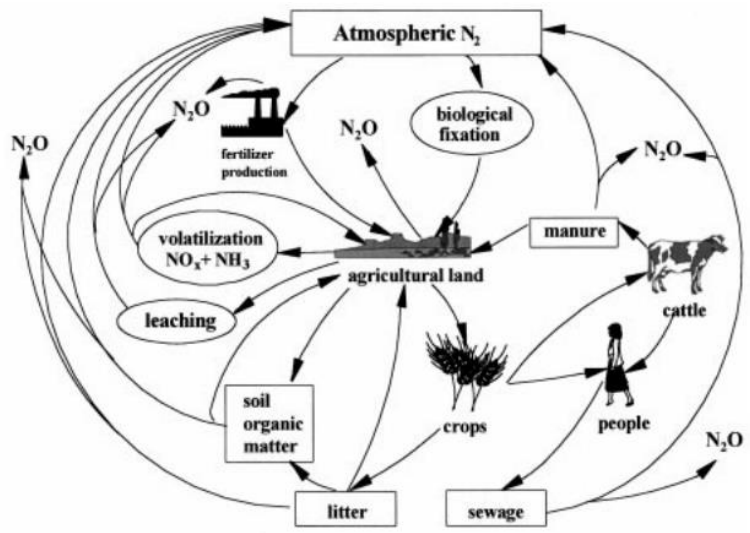

Figure 2.1 Depiction of the nitrogen cycle of agricultural soils and its relationship to N_2O production (Nevison et al., 1996).

2.3 Mitigation strategies to reduce ammonia emissions from the national beef herd

Extending the grazing season is an abatement strategy to reduce NH_3 in two ways. By extending the grazing season, the housing period is shortened with less volumes of slurry and manure produced, hence reducing the total emissions from housing and storage (Webb et al., 2005). Compared to indoor feeding systems, where urine is usually deposited on concrete, collected, stored, and later applied to land, the urine excreted by grazing animals' infiltrates into the soil before substantial NH_3 emissions can occur and overall NH_3 emissions per animal are therefore less for grazing animals (Bittman et al., 2014).

In 2019, 90.6% of agricultural NH_3 resulted from dairy and non-dairy cattle, with animal housing and storage contributing 47.4% and the land spreading of manures 32.3%, of these emissions (EPA, 2021). Further reductions in NH_3 emissions are achievable through the adaptation of new technologies in housing/storage and field

application methods. Ammonia emissions can be reduced from slurry during storage following the formation of a crust (Hyde et al., 2003). Misselbrook et al. (2005) observed reductions of NH_3 emissions of 50% from the formation of a natural crust. The addition of media to promote crust formation such as peat, zeolites (Portejoie et al., 2003) and straw has been shown to reduce NH_3 emissions (C. VanderZaag et al., 2009). Ammonia production is a function of pH and temperature therefore; the acidification of slurry has the potential to reduce NH_3 emissions from housing, storage and field application (Kai et al., 2008). The acidification of slurry with sulphuric acid resulted in lower NH_3 emissions with higher reductions in NH_3 achieved at lower pH (Petersen et al., 2014; Dai and Blanes-Vidal, 2013). Processing of manure with the separation of slurry into liquid and solid fractions has been shown to reduce NH_3 emissions (Dinuccio et al., 2012; Nyord et al., 2012) and increase N fertiliser value (Frost et al., 2009).

2.3.1 Timing and method of slurry application

The timing and method of slurry application has an effect to the extent of N loss as NH_3. Managing the timing of application to exploit optimum weather conditions has the potential to decrease total NH_3 emissions from slurry application using the splash plate (Lalor and Lanigan, 2010a). Previous analysis has shown that during the calendar year, the months from May to August present the greatest risk for increased NH_3 emissions in Ireland (Lalor and Lanigan, 2010b). Ammonia emissions can be further reduced by the application of slurry in narrow bands (trailing hose) (Sommer et al., 1997), trailing shoe (Webb et al. (2005), and injector (Mulder and Huijsmans, 1994). However, policy measures may need to be implemented to achieve significant levels of uptake in applications like trailing hose and trailing shoe, as the associated costs with these techniques are not offset by N fertiliser gains (Lanigan et al., 2017).

2.4 EU Climate Legislation

Ireland, as well as other EU countries, is bound by international legislation governing GHG emissions (Kyoto Protocol, S.I.244, 2006), trans-boundary gasses (Gothenburg Protocol, S.I.10, 2004) and water protection (Good Agricultural Practice for the Protection of Waters, S.I.610, 2010).

2.4.1 Ammonia legislation

Ireland is Party to the Convention on Long Range Transboundary Air Pollution (CLRTAP), through which the Gothenburg Protocol sets out targets for the control of certain air pollutants including NH_3, which are responsible for acidification, eutrophication and ground-level ozone pollution which leads to significant negative impacts on human health and the environment. In the EU, the implementation of the Gothenburg Protocol is achieved through the enforcement of the National Emissions Ceilings (NEC) Directive (2001/ 81/EC) (EU, 2017). Under Article 4.1 of the NEC Directive, Ireland is obliged to reduce and limit its annual national emissions of NH_3 by 116 kilo tonnes (kt) by 2010 and in each year thereafter (DAFM, 2019).

The Gothenburg Protocol was reviewed in 2012, has repealed, and replaced the emission ceilings under the current Protocol with national emission reduction commitments to be achieved by 2020 and beyond. This will compel Ireland to reduce its NH_3 of 1% below 2005 emissions by 2020 to 112 kt.

At the same time, the NEC Directive was reviewed under the EU Clean Air Package. In December 2016, the new Directive 2016/2284/EU was signed into European law, which repeals and replaces Directive 2001/81/EU. The amendment to the NEC directive will impose further reduction commitments for NH_3 of 1% below 2005 for

the period 2021 to 2029 and 5% below 2005 for the period beyond 2030 setting a new target of 107 kt.

2.4.2 Greenhouse gas legislation

The Kyoto Protocol is an international agreement linked to the United Nations Framework Convention on Climate Change (UNFCCC), under which, Ireland is committed to limiting GHG to 13% above baseline 1990 levels between 2008 and 2012 to 58.53 Mt CO2eq. Greenhouse gas emissions for Ireland in 2012 was 5.68 Mt CO_2 eq below the Kyoto threshold set (DAFM, 2017). In 2012, the Kyoto Protocol was reviewed which comprised of a number of amendments, termed the Doha Amendment, which was agreed by the UNFCCC and established a second commitment period for the years 2013-2020. In 2013, EU member states committed to jointly achieve a 20% reduction in their combined GHG compared to 1990 levels under the Climate and Energy Package 2008.

As part of the EU Climate and Energy Package for the post-Kyoto period 2013-2020, Ireland has also agreed to reduce national GHG emissions by 20% below 2005 levels by 2020 under the Effort Sharing Decision. Further to this, an Effort Sharing Regulation published in 2016 as part of the Paris agreement, proposed a 40% reduction in GHG target for Ireland for the period 2021 to 2030 relative to 2005 levels. However, these emission targets were adjusted downward for cost-effectiveness by 9%, providing a new annual GHG emission target for Ireland of 30% below the 2005 level by 2030. These targets will be a challenge for Ireland, not only as agriculture accounts for 37.1% of national emissions, but that agriculture represents 44% of Ireland's non-ETS emissions (Duffy et al., 2020) coupled with the ambitious plans proposed by the Department of Agriculture, Food and Marine, in Food Wise 2025 for the sector (DAFM, 2019).

2.5 Digestion, metabolism, and excretion of nitrogen in beef cattle

Nitrogen use efficiency in beef cattle is low at 10-20% (Cole and Todd, 2009) compared to dairy cattle 25-34% (Huhtanen and Hristov, 2009), resulting in large amounts of ingested N being excreted in the urine and faces. In this section N digestion, metabolism and excretion will be discussed and factors that influence them.

2.5.1 Digestion and metabolism of nitrogen in the rumen

Ruminants have evolved a unique digestive system that involves a symbiotic relationship with a consortium of microorganisms consisting of bacteria, protozoa, and fungi (Czerkawski, 1986). This facilitates them to breakdown plant fibres (cellulose and hemicellulose), low-quality protein feeds and non-protein N to valuable nutrients later utilised by the animal (Dewhurst et al., 2000). Crude protein ingested by ruminants is made up of two main fractions, rumen degradable protein (RDP) which is degraded to yield NH_3 and rumen undegradable protein (RUP) which avoids degradation in the rumen and is digested in the small intestine (SI) (Broderick, 1996). Rumen degradable protein is degraded in the rumen to yield NH_3, (which is absorbed into portal blood where in the liver it's detoxified to urea that is either excreted via the kidneys or recycled through the saliva or across the rumen wall when supply is limited), and microbial protein that passes onto the SI (Patton et al., 2014). In the SI 50-80% of total absorbable protein is a result of microbial protein synthesis in the rumen (Storm and Ørskov, 1983). The N fraction entering the SI comes from three main sources; undegraded feed protein, microbial protein and endogenous protein from secretions and epithelial cells (Lapierre et al., 2006). These fractions are broken down into amino acids (AA) that are absorbed into portal blood, metabolized in the liver, and used for milk protein and body tissues and depending on supply are either

excreted in urine via the kidneys when in excess or recycled through the saliva (Lapierre and Lobley, 2001).

The initial step of protein degradation in the rumen involves the attachment of consortia of bacteria to feed particles followed by the hydrolysis by protease enzymes into peptides and AA (Brock et al., 1982). These peptides and AA are taken up by microbial cells (Bach et al., 2005) with peptides further degraded by peptidases to AA. Amino acids are further metabolised and incorporated directly into microbial protein, if energy is available in the form of carbohydrates (Broderick et al., 1989). When energy is limited, protein is deaminated to yield volatile fatty acids (VFA), CO_2 and NH_3 (Tamminga, 1979).

Solubility is a key factor in the degradability of proteins, with soluble proteins easier to break down compared to insoluble proteins (Broderick et al., 1989). However, the rate and the extent to which protein degradation occurs also depends on the structure of the protein (Bach et al., 2005). The presence and number of different bonds within a protein plays a significant role determining the degradation of proteins, with soluble proteins such as immunoglobulins with extensive disulphide bonds taking longer to degrade, compared to proteins with less complex tertiary structures (Broderick et al., 1989).

2.5.2 Role of protozoa in rumen nitrogen metabolism

Protozoa are the second largest group of microorganisms found in the rumen (105 - 106 per ml) but because of their larger size may equal that of rumen bacteria in total microbial biomass (Williams and Coleman, 1992). Ciliated and flagellated protozoa both exist in the rumen (Bohatier, 1991), with ciliates primarily the majority, of which there are two groups, the entodiniomorphid (oligotrich) and the holotrich protozoa (Williams, 1982). Protozoa predate on bacteria as a protein source (Williams and

Coleman, 1992), and are responsible for intraruminal N recycling (Firkins and Yu, 2006). However, holotrich protozoa have more predatory activity than entodiniomorphid (Belanche et al., 2012). Protozoa have a longer retention time in the rumen, 4 times that of bacteria (Tarakanow et al., 1984). In studies conducted with defaunated animals, NH3 concentrations were lower compared to faunated animals (Eugène et al., 2004), which can be attributed to increased efficiency of microbial synthesis (+27%) and increasing the duodenal flow of microbial protein (+30%) (Newbold et al., 2015). In addition, defaunation can modify the composition of the rumen bacteria (Belanche et al., 2012), which influences the AA profile of the duodenal protein supply (Ivan, 2008).

2.6 Dietary strategies to reduce nitrogen excretion

2.6.1 Crude protein and rumen degradable protein

Undigested protein is excreted in the faeces, while digested protein is either converted into animal tissue or milk protein or converted to urea in the liver and excreted in urine by the kidneys (Satter et al., 2002). The relationship between dietary CP or N intake and N excretion in the urine is far stronger ($R^2 = 0.74$) than faecal N ($R^2 = 0.21$) and milk N ($R^2 = 0.30$) (Mulligan et al., 2004) and strategies that reduce CP are generally associated with a reduction in urinary N output (Mulligan et al., 2004; Broderick et al., 2008; Whelan et al., 2012).

Reducing dietary CP from 18.8 to 16.1% has been shown to reduce urinary volume excreted by 2 L d^{-1} (Leonardi et al., 2003) and by 6 L d^{-1} when CP was reduced from 19.4 to 13.5% (Colmenero and Broderick, 2006). Whelan et al. (2011) found that replacing a portion of maize silage (MS) with GS increased total VFA and NH_3 concentration in the rumen, suggesting increased CP degradation and fermentable

organic matter supply where the GS based diet was offered. Da Silva et al. (2016) demonstrated that the level of CP fed (110 g CP DM^{-1} vs 130 g CP DM^{-1}) to finishing beef cattle can affect ruminal NH_3 concentrations, blood urea N and urinary urea N, with the highest values obtained in cattle fed 130 g CP DM^{-1}. Similarly, Reis et al. (2016) noticed that as the level of CP supplementation increased with cattle fed low quality forage the concentration of ruminal NH_3 concentration increased.

Cole et al. (2006) noticed no difference in performance between finishing cattle fed 11.5% CP and 13.0% diet during the last 56 d on feed. In addition, during this period, N excretion decreased 0.1 to 2.9 kg/animal and estimate N volatilization decreased by approximately 4.4 kg $animal^{-1}$. In dairy cattle, Broderick (2003) demonstrated that there was no difference between milk production at 16.7% and 18.4% CP, while there was a marked increase in N excretion with increased CP in the diet. The majority of the extra N in the manure was in the form of urinary N, indicating that protein demand for milk production can be supplied by microbial protein from RDP (Broderick, 2003).

The CP or digestible CP system has been the long-standing method used to evaluate the protein value of feeds for ruminants. While simple and relatively acceptable for most traditional diets, the system does however have its limitations in certain situations, particularly with diets rich in degradable N or when formulating diets for high producing animals. The protein value of a feed or diet is best measured by the amount of amino acids absorbed in the small intestine from the dietary protein incorporating that which has escaped degradation in the rumen, and the microbial protein synthesized in the rumen from the available N and energy. The concept was initially proposed by Miller (1973), and Burroughs et al. (1974) and has since been developed into the many different systems used in modelling protein supply in ruminant production systems across the world, including the PDI (protein truly

digestible in the small intestine system) (Jarrige, 1989), the DVE/OBE system (DVE, truly absorbed protein in the small intestine; OEB, degraded protein balance) (Tamminga, et al., 1994), the National Research Council (NRC, 2001) and the Agricultural and Food Research Council (Alderman and Cottrill, 1997).

While each of the aforementioned systems differ in elements and terminology, theoretically they are similar in that they calculate their protein value from feed protein that escapes rumen degradation and from microbial protein synthesised in the rumen (Theodoridou and Yu, 2013).

The PDI system, first published by Vermorel (1978) is the system widely adopted in Ireland when formulating the protein requirements of ruminants (O'Mara, 1996).

The PDI of the feed is the sum of two fractions:

PDIA: the dietary amino acids that are undegraded in the rumen, but truly digested in the small intestine.

PDIM: the microbial true protein that is truly digested in the small intestine.

Microbial protein is dependent on the availability of degraded N and fermentable energy in the rumen. Therefore, each feed has two potential PDIM values:

PDIMN: the amount of microbial protein that can be synthesised in the rumen from the degradable dietary N, when energy and other nutrients are not limiting

PDIME: the amount of microbial protein that can be synthesised in the rumen from the available energy, when N and other nutrients are not limiting. The total supply of PDI from the feed includes PDIA plus PDIM from either the available energy or the available N as shown by the following terms:

PDIN= PDIA + PDIMN

PDIE=PDIA + PDIME

Therefore, the particular feature of the system is that each feed is apportioned two protein values: PDIN and PDIE. The real value of the feed is the lower of the two or in situations where animals are just consuming forages only (grazed pasture or conserved forages). When forage diets are supplemented with one or more concentrate feeds, the higher of the two values is the potential value of the feed when accompanied with a suitable complementary feed.

Hence, when calculating the PDI value of a diet, the PDIN and PDIE values are summed separately, with the lower of the two sums, PDIN or PDIE the actual PDI value of the diet. Therefore, when calculating the PDI value of a diet, adhering to the PDIN requirements for a certain level of production/gain will ensure that degraded dietary nitrogen is not fed excess to requirements, and that microbial protein production is utilised in full when feedstuffs high in fermentable organic matter (starch and sugars; high PDIE values), are incorporated.

Moreover, PDIM (the microbial true protein that is truly digested in the small intestine) depends on the amount and availability of N and energy, supplied by structural carbohydrates (NDF) and non-fibre carbohydrates in the feed. Structural carbohydrates can be represented by NDF and has supplemental effects on microbial growth in the rumen. Additionally, the NDF content in feed affects the rate of carbohydrate digestion, which is the major factor controlling the amount of energy available for microbial growth in the rumen. A lower NDF content is accompanied by higher concentrations of non-fibre carbohydrates and CP. Crude protein favourably improves the efficiency of microbial growth as long as N is not limiting and protein is not used as a source of energy.

Improved N efficiency can be achieved by better diet formulation (Dinn et al., 1998), to meet but not exceed the RDP requirement of the rumen microbes (Kalscheur et al.,

2006), or reducing RDP while holding productive performance (Cyriac et al., 2008). As RDP increased from 6.8 to 11.0% of DM, N efficiency declined from 36.3 to 28.2%, increasing urinary N output leading to higher NH_3 emissions from manure (Kalscheur et al., 2006; Cyriac et al., 2008). Without any changes in energy intake, the microbial and total N flow from the rumen tended to decline as dietary RDP is reduced (Cyriac et al., 2009).

2.6.2 Energy

The main carbohydrates offered to ruminants are in the form of structural fibres, (neutral detergent fibre (NDF) and acid detergent fibre (ADF), with considerable use of non-fibre carbohydrates (NFC) (starch and sugars) (Lean et al., 2014). Replacing a portion of NDF with NFC may increase the rate of energy available to the rumen microbes and improve RDP capture (Cabrita et al., 2006). Altering the site of carbohydrate digestion can also have an impact on N balance in ruminants. For example, maize starch is more resistant to rumen degradation than that of barley starch (Herrera-Saldana et al., 1990), and Castillo et al. (2001b) observed a numeric improvement in the portion of ingested N recovered in the faeces and a reduction in the portion of N excreted in the urine when maize starch replaced barley starch, suggesting that circulating urea N was rerouted into the large intestine to support increased microbial protein synthesis. Whelan et al. (2012b) obtained similar results, where urinary excretion was reduced in early lactation dairy cows offered pasture by replacing rolled barley with ground maize. Kim et al. (2009) found that replacing corn in cattle diets with wheat barn or soya hulls increased the ruminal NH_3 concentrations and urinary excretion. Similarly, Hunerberg et al. (2013) found that replacing barley grain and canola meal with dried distiller's grains increased N excretion in growing beef cattle. Røjen et al. (2012) also reported that increasing hindgut fermentation in

lactating dairy cows with 1,500 g d^{-1} of oligofructose by abomasal infusion resulted in a shift in N excretion from urine to faeces.

In grazing studies, increased concentration of water-soluble carbohydrates in perennial ryegrass fed to late lactation dairy cows lead to increased efficiency of utilization of the grass in the rumen, resulting in more efficient use of feed N for milk production and reduced N excretion (Miller et al., 2001).

2.7 Urea recycling and urine N excretion

Urea is a major end product of N metabolism in ruminants (Merchen, 1993; Lapierre and Lobley, 2001). Rumen Degradable Protein that is fed in excess of microbial requirements for protein synthesis results in the formation of NH_3 that is absorbed and converted to urea in the liver (Parker et al., 1995; Lapierre et al., 2005) and transported in the blood (Spek et al., 2013b). With a reduction in N intake, the amounts of N digested and excreted, in particular via urine decreases, resulting in higher proportions of urea recycled to the gut (Marini and Van Amburgh, 2003; Røjen et al., 2008; Wickersham et al., 2008). With the decline of further systemic urea, this proportion of recycled urea increases until it becomes almost complete at extremely low N intake (Reynolds and Kristensen, 2008).

In a study by (Spek et al., 2013a) the gastrointestinal urea entry rate was 85% and 74% of the total urea entry rate in blood plasma when feeding a MS diet with 11.5% and 15.4% CP, respectively. Urea entry in the gastrointestinal tract was lower however (180 g versus 225 g urea-N d^{-1}, respectively) because of a lower urea pool in blood plasma (7.5 versus 12.6 mg urea-N dL^{-1}, respectively). Although conditions of N limitation can easily be met with maize-based diets, similar effects also have been observed with grass herbage harvested under extreme growing conditions (Warner et

al., 2012) where it was demonstrated that for diets including 85% grass herbage on DM basis, with CP content varying between 10.0% and 16.1% in herbage DM, rumen NH_3 concentrations varied between 0.7 and 10.2 mM L^{-1}.

Although a similar mechanism of urea recycling occurs in the large intestine, its rate seems to be of less importance as observed by Røjen et al. (2012) where 1.5 kg d^{-1} of oligofructose infused in the abomasum caused only a slight shift in apparent faecal N digestibility from 58% to 54.0%. This seems to imply that under normal feeding conditions, the large intestine has a relatively small influence on urea recycling and on the amount of urea excreted with urine. Similar results were observed by Gressley and Armentano (2007), with inulin infused into the abomasum resulting in increased faecal N and decreased urinary N, milk urea N and blood urea N (BUN).

2.7.1 Oscillating diets

To exploit this phenomenon, Cole (1999) put forward that N utilization could be improved with increased or optimized N recycling to the rumen if sufficient ruminal fermentable carbohydrates were available within the rumen. The theory was that by oscillating dietary CP at a rate similar to the rate of passage, the total intake of CP fed during the finishing phase would be reduced without adversely affecting animal performance. In order to optimize N recycling, the theory was to synchronize a ruminal N deficiency with excess N in the lower gut and similarly to synchronize any ruminal excess N with an N deficiency in the lower gut by oscillating the dietary CP percentages between deficient and adequate. Early studies indicated that oscillating dietary CP at 24 h had no effect on N retention whereas at 48 h increased N retention by 38.0% (Cole, 1999). However, in a subsequent study Cole et al. (2003) found no effect on N digestion and N retention when oscillating dietary CP.

Results of oscillating dietary CP in forage diets have been less promising in improving N retention (Simpson et al., 2001; Ludden et al., 2002a,b; 2003). In order for oscillating diets to increase N recycling to the rumen, oscillating diets need to be deficient in degraded intake protein or the average CP concentration needs to be near or below the animal's requirements (Cole and Todd, 2009).

Phase feeding is a practice rarely used in cattle feeding operations but more common in the swine and poultry industries. Trenkle, (2002) found that the level of CP can be reduced in finishing cattle diets with no effect on cattle performance. Cattle fed 11.5% CP diet compared to cattle fed 13.0% CP diet for the last 56 d of feed, had the same ADG, DMI. However, if cattle changed from the 13.0% CP diet to the 11.5% CP diet with less than 56 d to slaughter, they had lower ADG and DMI, indicating that an adjustment period maybe required (Cole et al., 2006).

2.8 Dietary additives

Research over many years has shown mixed results for N excretion and NH_3 emissions using a variety of dietary additives including plant tannins (Min and Hart, 2003), saponins (Hart et al., 2008), essential oils (Cobellis et al., 2016b), or dietary salt (Spek et al., 2013a).

The banned use of antibiotics and ionophores in animal feeds in the European Union (Regulation 1831/2003/EC; European Union, 2003) has intensified research in the pursuit for alternative natural products that can modify ruminal fermentation (Castillejos et al., 2005). To date, a substantial amount of research has focused on the role secondary plant compounds (essential oils, saponins, and tannins) play in modifying ruminal fermentation (Wallace et al., 2002; Patra and Saxena et al., 2011; Cobellis et al., 2016a).

2.8.1 Tannins

Tannins are phenolic secondary compounds in plants, which are widely distributed in forages, legumes, cereals, grains, trees, and shrubs (Patra and Saxena, 2011; Min et al., 2004). Tannins are classified as either hydrolysable or condensed with some plants containing both, but generally most plants predominantly contain one form or the other (Makker, 2003; Patra and Saxena, 2011). Condensed tannins are of interest in ruminant nutrition because they are the main tannin found in forages and legumes and because of their ability to form tannin-protein complexes in the rumen. They inhibit the growth and activities of proteolytic bacteria and have been shown to shift the site of N metabolism from the rumen to the lower digestive tract and large intestine (Min et al., 2004; de Klein and Eckard, 2008).

Condensed tannins directly supplemented to ruminants (Carulla et al., 2005; Krongberg and Liebig, 2011; Taha et al., 2015) or indirectly with forages known to contain high levels of tannins (Misselbrook et al., 2005; Grainger et al., 2009; Hymes-Fecht et al., 2013; Wischer et al., 2014) decreased ruminal NH_3 and BUN with less protein degraded in the rumen, shifting N excretion from urinary to faecal and increasing overall N utilization.

2.8.2 Saponins

Saponins are secondary compounds produced by plants but also by lower marine animals and some bacteria (Riguera, 1997; Yoshiki et al., 1998; Patra and Saxena, 2009). Saponins get their name from their ability to form soap-like foams in aqueous solutions (Hart et al., 2008). The primary mode of action of saponins appears to be their ability to inhibit/removal of protozoa (defaunation) in the rumen, which in turn increases the efficiency of microbial protein synthesis and enhances the flow to the SI (Hristov et al., 1999; Wallace, 2004; Hart et al., 2008; Patra and Saxena, 2009;

Wanapat et al., 2015). However, while many saponins have been shown to decrease ruminal NH_3 concentrations, across a number of studies these results are inconsistent (Jouany and Morgavi, 2007; Hart et al., 2008). Over time the efficiency of saponins decreases, not because of protozoa adaptation but due to rumen bacterial population degrading the saponins (Patra and Saxena, 2009; Patra, 2012).

2.8.3 Essential Oils

Essential oils (EO) have been examined in ruminant diets *in vitro* due to their antibacterial properties and have been shown to affect ruminal N metabolism depending on the supplemented dose (Castillejos et al., 2005). Busquet et al. (2006) showed that some EO reduced NH_3 concentration at high concentrations (3000 mg L^{-1}) but that the effects were marginal at moderate doses (300 mg L^{-1}) and non-existent at low doses (3 mg L^{-1}). Coupled with decreased ruminal NH_3 concentrations, there was a reduction in total VFA concentration suggesting a reduction in overall rumen fermentation (Cobellis et al., 2016a). The principal source of energy for ruminants are the VFA, therefore decreasing ruminal VFA production could have nutritional consequences if this effect were expressed *in vivo* (Benchaar et al., 2008).

2.8.4 Black seed oil

Black seed (black cumin, *Nigella sativa*) oil (BO) is an herbaceous plant growing in Asian and Mediterranean countries (Kumar et al., 2005). Black seed oil is used as a spice, condiment, carminative, food preservative, as well as a protective and curative treatment for numerous disorders in traditional and Indian folk medicine. It is known to have antimicrobial and strong antioxidative properties (Burits and Bucar 2000; Ahmad et al., 2013). The black seeds contain 36-38% fixed oil, with proteins, alkaloids, saponins, and EO making up the rest of the composition (Kumar et al., 2005). There is a paucity of information available on the effects of BO in animal

nutrition (Sahinler et al., 2005; Canogullari, 2009; Klevenhusen et al., 2015). In their *in vitro* study Klevenhusen et al. (2015) found that supplementing with BO at 50 mg L^{-1} and 500 mg L^{-1}, that both levels significantly reduced NH_3 concentration. El-Naggar et al. (2017) found that supplementing different levels of BO to growing lambs increased digestibility of DM, organic matter (OM), CP, and reduced ruminal NH_3 concentrations.

2.8.5 Salt supplementation

Ammonia emission is a process driven by NH_3 concentration differences between the NH_3 in the urine or manure surface exposed to air, and very sensitive transport of air above these surfaces (Sommer et al., 2006). This makes the urea concentration in excreta an important determinant in the rate of NH_3 emissions (Ndegwa et al., 2008). Urea concentration depends on both the amount of urea excreted with urine and the volume of urine produced (Symonds et al., 1981). Urine volume strongly depends on the number of solutes excreted with urine, and a strong relationship was obtained with the amount of sodium and potassium excreted and to a lesser extent with the amount of N excreted with urine (Bannink et al., 1999). Recent balance trials including dietary salt (sodium chloride; NaCl) addition confirmed this relationship (Spek et al., 2012, 2013a), were urine N concentration with control diets was well within the general range of N concentration 10 g kg^{-1}, whereas additional NaCl reduced urinary N concentrations to as low as 3 g kg^{-1}. Grass and leguminous forages have a relative high content of cations and high buffering capacity (i.e. cation exchange capacity), compared to a maize crop (Giger-Reverdin et al., 2002) leading to twice as much urine produced. Therefore, N excretion mitigating measures such as replacing GS with MS may be expected to lower urea excretion rate, but it may be accompanied by a

concentrating effect on urine urea due to a lower volume of urine produced and by less frequent urinations.

2.8.6 Chitosan

Chitosan (*N*-acetyl-D-glucosamine polymer) is a natural biopolymer formed from the deacetylation of chitin (Belanche et al., 2016c). Chitin is the second most abundant organic compound on earth next to cellulose, is found in the cell wall of lower plants and the exoskeletons of some arthropods and crustaceans and fungi (Dias et al., 2017). Chitosan is obtained by deacetylating chitin with NaOH (Kong et al., 2010) and in doing so increases the antimicrobial activity as well as the solubility aqueous media. Composed of polymers of D-glucosamine and N-acetyl-D-glucosamine units, it is the ratio between these two units that defines the degree of acetylation or deacetylation in chitosan, which along with the molecular weight play an important role in the antimicrobial activity of chitosan compound (Verlee et al., 2017). For this reason, chitosan should not be considered as a single compound but rather a series of different compounds, which differ in degree of acetylation and other physiochemical characteristics.

Due to its biodegradability and non-toxic properties, chitosan has received much attention for its diverse applications in medicine and food preservation because of its antimicrobial properties (Cuero, 1999; Shahidi et al., 1999; Jeon et al., 2002). The antimicrobial activity of chitosan is influenced by several factors: pH, type of microorganism, molecular weight, and degree of deacetylation (Kong et al., 2010). While the exact mode of action of chitosan is contested, the most widely accepted theory is that the polycationic nature of chitosan due to the positive charges of the protonated amino groups (NH_3^+) of chitosan, maybe the significant attribute that allows it to interact with the negatively charged outer membrane of numerous micro-

organisms, causing extensive alterations to the cell surface, leading to leakage of intracellular substances that results in cell death (Ma et al., 2017). The rumen microbiome, vital to the ruminant in its ability to utilize low quality fibrous feeds and produce high quality protein products. In the past, several nutritional strategies have been evaluated to improve rumen fermentation while simultaneously reducing the environmental impact from ruminants. The rumen is home to at least 30 predominant bacterial species, with estimated total concentration of 10^{10} to 10^{11} per ml of rumen fluid (Miron et al., 2001). Previous studies demonstrated the positive antimicrobial, anti-oxidative, immunoregulatory effects of supplementing pigs and poultry diets with chitosan such as enhanced nutrient digestibility and growth performance (Swiatkiewicz et al., 2015; Guan et al., 2019). However, its use in ruminant nutrition has not been extensively investigated, with studies to date report conflicting results (Araújo et al., 2015). When evaluated *in vitro* using the rumen simulation technique (RUSITEC), Goiri et al. (2009) found that including chitosan at 1971 mg L^{-1} reduced NH_3 concentration by 46%. However, in a batch fermentation study chitosan had no effect on fermentation parameters (Belanche et al., 2016c) while tended to increase NH_3 concentration 2 h post feeding (Belanche et al., 2016b). Moreover, several *in vivo* studies have demonstrated the effects of chitosan inclusion on ruminant digestion and fermentation to be inconsistent (Goiri et al., 2010; Araújo et al. 2015; Mingoti et al., 2016; Vendramini et al., 2016; De Pavia et al., 2017; De Valle et al., 2017; Dias et al., 2017). For example, apparent tract digestibility of DM, CP and NDF increased with chitosan inclusion (Araújo et al. 2015; Mingoti et al., 2016; Vendramini et al., 2016; De Valle et al., 2017; Dias et al., 2017) while others reported negative effects (De Pavia et al., 2017; Zanferari et al., 2018). Similarly, results on rumen fermentation parameters remain equivocal. Goiri et al. (2010) demonstrated that chitosan had the

ability to shift rumen fermentation to more an efficient route while simultaneously reducing ruminal NH_3. While other studies showed that chitosan inclusion could affect ruminal N metabolism in a dose-dependent manner (Araújo et al. 2015; Dias et al., 2017), thus warranting further investigation on this topic.

2.8.7 Seaweeds

In some countries, seaweeds have been used to feed livestock for centuries, especially along coastal areas, and in times with reduced fodder stocks, seaweeds were grazed by livestock along beeches or dried, stored in barns and fed for 6-8 weeks of the year (Evans and Critchley, 2014).

Seaweeds contain a broad spectrum of nutritional compounds, polysaccharides, polyunsaturated fatty acids, and polyphenols (such as phlorotannins (PT), bioactive peptides, vitamins, and minerals (Kumari et al., 2010; Tierney et al., 2010; Fitzgerald et al., 2011).

They are divided into three different groups on the basis of pigmentation: brown algae (Phaeophyceae), red algae (Rhodophyta), and green algae (Chlorophyceae). Furthermore, they differ considerably in size, shape and composition of bioactive compounds such as photosynthetic pigments, storage compounds, composition of cell walls, and as a result differ in their chemical composition. Variation in chemical composition also exists within species due to factors such as harvest season, light, water temperature and habitat. While brown and red algae are predominantly found in marine waters, green algae are also common in freshwater rivers and lakes (Makkar et al., 2016).

On a DM basis, red and green seaweeds tend to have a higher CP content (18-50% DM) compared to the moderate levels in brown seaweeds (5-12% DM) making them

an ideal protein source. The main carbohydrate in brown seaweeds is laminarin, a polysaccharide of glucose, whereas, in red seaweeds the main storage compound is starch. Red and brown seaweeds differ in their compositions of bioactive compounds with PT a polyphenolic compound found exclusively in brown seaweeds. Phlorotannins are less complex than terrestrial tannins and are produced entirely by polymerization of phloroglucinol units (1,3,5-trihydroxybenzene) linked together through aryl aryl, diaryl-ether or diaryl-diether bonds and biosynthesised by the acetate-malonate pathway (Arnold and Targett, 1998). Tannins are known to have an ionic binding ability to protein and fibre, with the affinity towards protein being the highest (Min et al., 2004), and as a result, ruminal fermentation of protein decreases. Terrestrial tannins found in legumes and forages have been studied extensively because of their ability to form tannin-protein complexes in the rumen (Patra and Saxena, 2011). They inhibit the growth and activities of proteolytic bacteria and have been shown to shift the site of N metabolism from the rumen to the lower digestive tract and large intestine (Min et al., 2004; de Klein and Eckard, 2008). The effect of PT on rumen metabolism is still in its infancy and studies to date have produced conflicting results.

Phlorotannins from *Ascophyllum nodosum* induced a reduction in NH_3 concentrations *in vitro* (Wang et al., 2008, 2009) with similar results obtained with extracts from *Laminaria digitata* (Vissers et al., 2018). Belanche et al. (2016a) found -24% reduction in N degradability with seaweed meal from *Ascophyllum nodosum* included at 50 g kg DM^{-1}. Including *Saccharina latissimi* at high levels (250 g kg DM^{-1}) did not affect rumen fermentation parameters *in vitro* (Maia et al., 2019). Molina -Alcaide et al. (2017) observed no difference in NH_3 concentration *in vitro* among *Alaria esculenta*, *Laminaria digitata*, and *Pelvetia canaliculate*, however, the time of year

that the seaweeds were harvested affected NH_3 concentrations, with lower values observed with seaweeds harvested in the autumn. Therefore, more research in this area is required.

2.9 Methods of evaluation used

2.9.1 RUSITEC

In vitro continuous-culture fermentation studies that involve the incubation of substrates with rumen fluid and simulate the rumen environment have been widely used to allow researchers to evaluate the effects of diet, feed composition, and feed additives on ruminal digestion, microbial protein synthesis, and ruminal fermentation, and CH_4 production (Hoover et al., 1976; Benchaar et al., 2008; Belanche et al., 2016a). Depending on the research question, *in vitro* studies can be valuable for screening and informing on the suitability for further evaluation *in vivo* (Yáñez-Ruiz et al., 2016). However, results from *in vitro* studies should be interpreted cautiously as a positive outcome *in vitro* does not guarantee that the identical treatment will have a similar effect *in vivo*. The main advantages of these techniques are: (1) the capacity to evaluate a large number of dietary treatments, in sufficient replication, over a short period of time; (2) to investigate higher feed levels/dose rates of a given feed additive; (3) avoids ethical issues involving the use of live animals, and; (4) they are less expensive compared to *in vivo* studies (Hristov et al., 2012). Another major advantage of a continuous-culture system, compared to a batch-culture *in vitro* system, is the ability to remove fermentation end products and maintain a relatively stable fermentation for prolonged periods (Czerkawski and Breckenridge, 1977). Among the continuous culture fermentation systems, the RUSITEC is an established method to simulate and to investigate rumen microbial processes *in vitro*, while avoiding

variability associated with animals in a standardized environment (Czerkawski and Breckenridge, 1977). However, due to a variety of inherent characteristics fundamental with all *in vitro* systems, the original microbial community may degenerate in the RUSITEC system and protozoa disappear (Slyter and Putnam, 1967; Mansfield et al., 1995), although some designs are able to better maintain microbial and protozoal diversity (Teather and Sauer, 1988; Muetzel et al., 2009). Generally, data obtained through RUSITEC systems is lower compared to similar diets *in vivo*, with many important differences existing between both. Hristov et al. (2012) evaluated studies with RUSITEC, and *in vivo* data published simultaneously. Overall, the authors found that total VFA concentrations observed in RUSITEC systems were 67% of the total VFA concentration observed *in vivo*. Furthermore, acetic acid concentrations were 90% of acetic acid concentrations observed *in vivo*, with lower results observed for propionic acid concentrations but similar concentrations of butyric acid concentrations. Nutrient digestibility of OM and NDF were lower in the RUSITEC data compared with *in vivo*; with the average NDF digestibility 35% lower than that observed *in vivo*, which would partially account for the differences in VFA concentrations. However, when interpreting these results, it is important to note that the RUSITEC system is designed to simulate the rumen and not the total digestive tract.

2.9.2 Metabolism studies

Ruminant livestock are inefficient utilizers of feed N. Nitrogen use efficiency in beef cattle is low at 10-20% (Cole and Todd, 2009) compared to dairy cattle 25-34% (Huhtanen and Hristov, 2009), with a conversion of N into milk 27% or body weight gain 14% of for dairy farms and beef operations respectively (Hristov et al., 2011). Nitrogen that is not retained is excreted in urine and faeces, which to leads to growing

environmental concerns around water pollution and gaseous N emissions (Kulling et al., 2001; Hristov et al., 2011; Cameron et al., 2013). The large variation in urinary N excretion compared with faecal N, presents an opportunity to manipulate diets to reduce urinary N excretion (Dijkstra et al., 2018). Therefore, understanding N metabolism and studying processes and practices that can improve the efficiency of N utilization for productive purposes and thus, reduce the environmental impact from ruminants (Bergen, 2007; Schwab and Broderick, 2017).

Using the most accurate and precise measurement techniques is critical for obtaining reliable experimental results on N utilization by ruminants. Experimental design is one of the most critical aspects in any animal experiment. The design used in animal experiments usually falls within two categories: continuous/long-term design and crossover design/short-term. Continuous design experiments allow for the evaluation of treatment effect on variable over a longer duration. One of the main disadvantages with this design is that variability among individual animals can be high, requiring large numbers per treatment. Whereas, with the crossover design, once the design is complete, every animal receives every treatment and acts as its own control. Consequently, the statistical power with this type of design is almost always greater than a comparable number of experimental units in that of a continuous design because animal variation is accounted for. One disadvantage with crossover designs is that the duration is short, and animals may change their nutrient metabolism during the course of the experiment. Another disadvantage is the potential of carryover effects from the previous treatment. However, carryover effects can be minimised by using designs balanced for carryover effects, extending the experimental period, and using wash-out periods.

Nitrogen balance studies have long been reported in the literature for ruminants and they provide an insight to N retention or loss. Nitrogen balance is calculated as the difference between N intake and the sum of losses from the body, predominantly urine, faeces, and milk. Accurate measurements of N intake, urinary and faecal N excretion are essential for estimating N balance. Rymer (2000) recommended experimental periods of 4 to 12 d to account for the variation in faecal output. The collection feed samples should begin 24 to 48 h before the collection of faeces (Spanghero and Kowalski, 1997), to allow for the passage of undigested feed to be excreted in the faeces. Additionally, animals offered TMR diets *ad libitum*, selection of the feed ingredients can occur, resulting in differences in the composition of the diet and the refusals. Therefore, the collection and analysis of the diet offered, and refusals is required (St-Pierre and Weiss, 2015). In most metabolism studies, measurement of N in urine and faeces usually involves the restraining of the animal in a metabolism stall, which facilitates the total collection of N excreted, involving separate collection of faeces and urine over a 24 h period. To prevent the loss of volatile N in urine from the hydrolysis of urea by urease, urine samples need to be collected with minimal contamination from faeces and acidified to pH 2.0. Faecal samples should be analysed fresh compared to air or freeze-drying which can incur N losses of 15.0% (Spanghero and Kowalski, 1997). All samples should be frozen immediately or treated with a preservative to minimise microbial degradation of N prior to analysis.

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Chapter 3

Effect of supplementing grass silage based diets with different concentrate carbohydrate sources with different fermentation profiles, on nitrogen metabolism of beef heifers fed to maintenance

Published in Ruminants, April 2022

3.1 Abstract

The synchronous supply of energy and nitrogen (N) substrates to the rumen microbes on grass silage (GS) based diets can potentially lead to reduced levels of N excreted in the urine. The objective of this study was to evaluate the effect of supplementing GS based diet with carbohydrate sources differing in rumen fermentation profile on N metabolism of beef heifers. Six Belgian Blue × Holstein Friesian cross beef heifers (487 ± 29 kg BW) were used in a 3 × 3 Latin Square design (n = 6). Dietary treatments were: (RB) GS supplemented with rolled barley; (MM) GS supplemented with maize meal and (SH) GS supplemented with soya hulls offered at 40: 60 forage to concentrate ratio on a dry matter (DM) basis, at maintenance feeding (40g DM kg^{-1} $BW^{0.75}$). Carbohydrate source had no effect on DM, organic matter, or N intake or total N excretion and the amount of N excreted in the urine ($P > 0.05$). Animals offered MM excreted a higher percentage of N in the faeces and a lower percentage of N in the urine compared to animals offered RB ($P < 0.05$). There was a treatment x time interaction for ruminal ammonia (NH_3) concentrations ($P < 0.01$). Ruminal NH_3 concentrations peaked at 2 h post-feeding for all treatments. At 3 h post-feeding ruminal NH_3 concentrations for the RB treatment remained higher compared to MM and SH treatments. Individual and total ruminal volatile fatty acids were similar among dietary treatments ($P > 0.05$). Supplementing GS based diets with different carbohydrate sources had no impact on the total level of N excreted or the amount of N excreted in the urine. However, there was a higher percentage of N excreted in the faeces and a lower percentage of N excreted in the urine when animals were offered MM compared to those offered RB ($P < 0.05$).

3.2 Introduction

Globally, there are growing concerns as levels of ammonia (NH_3) in the atmosphere continue to rise (Warner et al., 2017) arising from excess nitrogen (N) excreted from agriculture (Aneja et al., 2020, Zeng et al., 2018) which accounts for 93% of total NH_3 emissions within the European Union (EU) (EEA, 2020). Ammonia when redeposited increases acidification and the eutrophication of terrestrial and aquatic ecosystems (Hristov et al., 2011b). Furthermore, NH_3 reacts with atmospheric acids to form secondary particles, (particulate matter, $PM_{2.5}$) which contribute to air pollution, estimated to be responsible for 4.2 million premature deaths worldwide in 2016 (WHO, 2019). In Ireland, agriculture accounts for 99.2% of total NH_3 emissions, with cattle responsible for 90% of this total, with animal housing/storage and land spreading of manures accounted for 47.1%, with deposition at grazing accounting for 12.3% (Duffy, 2019).

Beef cattle are inefficient in utilising N, only retaining 10-20% of N consumed (Cole and Todd, 2009) resulting in large amounts of ingested N being excreted in urine and faeces. Reducing urinary N excretion is more favourable, as the rate of volatilisation of urinary urea N to NH_3 is much faster compared to the organic N compounds in faeces, (Jarvis et al., 1989, Varel et al., 1999). This problem is particularly relevant to Ireland where the main livestock production systems are pasture based, with limited supplementary feeding for much of the year (Lanigan et al., 2017). Typically, in an Irish suckler calf to beef system, pasture, grass silage (GS) and concentrates make up 66.0%, 27.0% and 7.0% respectively of feed dry matter intake (DMI) annually (McGee et al., 2006), with barley the traditional concentrate carbohydrate source (Drennan et al., 2006).

Grass silage is the main conserved forage fed to beef cattle in Ireland. During the ensiling process, water-soluble carbohydrates are the primary fermentation substrate and plant proteins are broken down to amino acids and NH_3, the extent of which is dependent on the rate of pH decline (Kung Jr, 2001). Therefore, the main carbohydrate substrates available for fermentation in the rumen are slowly fermented fibre substrates, cellulose and hemicellulose, while the N compounds in GS are mainly soluble leading to instant degradation within the rumen (Hersom, 2008). This asynchronous release of energy and N components in the rumen has been considered an important cause of the low N use efficiency for microbial growth observed with diets such as GS (Van Vuuren et al., 1993). The incorporation of cereal grains in concentrate feed formulations can provide an energy source in the form of starch to the rumen microbes, thus allowing a greater capture of N in the rumen (Lardy et al., 2004).

Globally, 36% of cereal grains are used for livestock feed (FAO, 2019), however, the inclusion of by-products in livestock feeds is increasing in Ireland, with imports of maize and soya hulls increasing from 925,000 to 1,110,000 tonnes, and 350,000 to 400,000 tonnes respectively between 2015 and 2017 (CSO, 2019). The starch found in wheat, oats, and barley is more rumen degradable than the starch in maize (Nocek and Tamminga, 1991). Castillo et al. (2001) observed that when maize starch replaced barley starch in the diet, there was an improvement in the portion of ingested N recovered in the faeces and a reduction in the portion of N excreted in the urine, suggesting that circulating urea N was rerouted into the large intestine to support increased microbial protein synthesis.

Soya hulls contain a variety of energy substrates for ruminal microbes, including non-fibre carbohydrates and a highly digestible neutral detergent fibre (NDF) fraction

(Trater et al., 2001). Contrasting results have been found in the ruminal NH_3 concentration when soya hulls replaced grains in the diets of dairy cows (Ipharraguerre and Clark, 2003). However, when soya hulls replaced barley as the energy source in the concentrate offered to growing cattle fed grass silage, performance parameters were not affected (Lenehan et al., 2015).

It was hypothesised that offering a carbohydrate that is rapidly degraded within the rumen, will in turn capture more N within the rumen and reduce N excretion.

Therefore, the objective of this study was to evaluate the effect of supplementing grass silage based diets with concentrate carbohydrate sources with different fermentation profiles on N metabolism of beef heifers fed to maintenance.

3.3 Materials and methods

All procedures described in this experiment were approved by the Animal Research Ethics Committee (AREC) at University College Dublin (UCD) and conducted under experimental license from the Health Products Regulatory Authority (HPRA) (approval number: AE18982/P083) under the European directive 2010/63/EU and S.I. No. 543 of 2012. Each person who carried out procedures on experimental animals during this experiment were authorised to do so by means of individual authorisation from the HPRA. This experiment was conducted at UCD Lyons Research Farm, Celbridge, Naas, Co. Kildare, Ireland, W23 ENY2 (53^0 17' 56" N, 6^0 32' 18" W).

3.3.1 Experimental design and dietary treatments

Six beef heifers (*Bos taurus* strain Belgian Blue × Holstein Friesian) with an initial body weight of 487 ± 29 kg were surgically fitted with permanent ruminal cannula (100 mm i.d.) (Bar Diamond Inc. Idaho, USA) and assigned to one of three dietary treatments in a replicated 3 × 3 Latin Square design (n = 6). Dietary treatments were

as follows: RB) GS supplemented with rolled barley; MM) GS supplemented with maize meal and SH) GS supplemented with soya hulls offered at 40: 60 forage to concentrate ratio on a dry matter (DM) basis. All diets were formulated to be isonitrogenous and balanced with soya bean meal (Table 3.1). Diets were offered at maintenance (40 g DM kg^{-1} $BW^{0.75}$) (Jarrige, 1989) twice daily as a total mixed ration (TMR) at 0800 and 1600 h using a Calan Data Ranger (American Calan, Northwood, New Hampshire, USA). The GS used during this experiment consisted of predominantly perennial ryegrass (*Lolium perenne L.*). The crop was felled during the early boot stage of vegetation (growth stage 410; (Zadoks et al., 1974), wilted for 16 h, baled, and wrapped using a McHale Fusion 3 Integrated baler/wrapper (McHale, Ballinrobe, Co. Mayo, Ireland). The crop was ensiled without the use of an additive. Prior to the commencement of the experiment, samples of silage were collected for subsequent chemical analysis (Agri-Food and Biosciences Institute, AFBI-Hillsborough, Co. Down, BT26 6DR) using NIRS (FOSS NIR systems 5000 [FOSS UK, Warrington, Cheshire, UK]).

Table 3.1 Ingredient composition and chemical composition of dietary treatments.

	Diet[1]		
Ingredient composition (kg DM^{-1})	RB	MM	SH
Rolled barley	3.0	-	-
Maize meal	-	3.0	-
Soya hulls	-	-	3.0
Soya bean meal	0.77	0.94	0.77
Grass Silage	1.47	1.47	1.47
Barley Straw	1.0	1.0	1.0
Mineral premix[2]	0.10	0.10	0.10
Chemical composition (g kg DM^{-1})			
Dry matter (g kg^{-1})	44.72	44.12	44.01
Crude protein	13.45	13.33	13.62
Starch	17.14	19.09	0.67
Neutral detergent fibre	30.35	28.99	49.63
Acid detergent fibre	16.85	16.36	32.49
Ash	6.37	6.70	7.15
Ether extract	1.77	1.44	0.87
Gross energy (MJ/kg DM)	15.22	15.31	15.14

[1]Grass silage based diet supplemented with rolled barley (RB), maize meal (MM) or soya hulls (SH).

[2] Vitamins/Minerals consists of the following: Calcium Carbonate, sodium chloride, mono-dicalcium, phosphate and (sugar) beet molasses. Additives per kg: Vit A (retinol) (E672) 200000 IU, Vit D3 (cholecalciferol) (E671) 40000 IU, Vit E (ali-rac-aipha-tocopheryl acetates) (3a700) 1000 IU.Compounds of trace elements: Basic cobalt (II) carbonate monohydrate (3b302) 175 mg, Copper sulphate pentahydrate 12000 mg, ferric oxide (E1) 4790 mg, calcium iodate anhydrous (E2) 794 mg, manganous oxide (E5) 3226 mg, Zinc oxide (E6) 5556 mg, sodium selenite (E8) 100 mg. Constituents: crude ash 96%, calcium 20 %, Phosphorus 2%, sodium 15%, Copper added 3000 mg, selenium added 45 mg.

Each experimental period consisted of a 14 d dietary adjustment period where the animals were fed their respective diets using a Calan Broadbent controlled feeding system (American Calan, Northwood, New Hampshire, USA) followed by an 11 d experimental period where the animals were housed in metabolism stalls (1.4 × 1.8 m). During this period in the metabolism stalls, animals were allocated the first 3 d for acclimatization, followed by 8 d to facilitate an N-balance study, rumen sample collection and *in sacco* DM degradability determination. While in the metabolism house, each animal was assigned to their own individual stall for the duration of the experiment with *ad libitum* access to water.

3.3.2 Data and sample collection

During the N-balance study, all animals were fitted with a specially constructed harness system to facilitate the separate collection of urine and faeces. This allowed the urine to flow through a plastic pipe into a plastic container, which contained 150 ml of 250 ml L^{-1} sulphuric acid to prevent microbial degradation and the loss of volatile N as NH_3. Total faeces were collected in trays behind each animal. Urine and faeces were weighed daily following morning feeding.

Samples of concentrates (rolled barley, maize meal, soya hulls and soya bean meal) were collected weekly, while GS and TMR samples were collected daily later pooled per treatment and per animal for each experimental period. Samples were dried at 55 °C for 48 h for chemical analysis with additional samples frozen and stored at -20 °C for later total N analysis. Each morning during the N-balance study, faeces were mixed per animal and a 5% faecal sample collected. These samples were then split; a 10.0% subsample was taken and frozen to -20 °C for subsequent chemical analysis with the remaining sample dried at 55 °C for 240 h in a forced air oven. Dried and fresh samples of faeces were composited on an animal basis for each N-balance study. Urine samples

were collected each morning with 2.5% volume sample taken and immediately frozen to -20 °C.

On d 1 and 5 of each N-balance period, blood samples were collected by jugular venepuncture at 1600 h prior to pm feeding into blood collection tubes containing Lithium Heparin (REF: 367526, BD-Plymouth, UK), centrifuged at 1,600 × *g* for 20 minutes at 4 °C for plasma extraction. These samples were then stored at -20 °C pending analysis for plasma urea N, total protein, and creatinine concentrations.

In sacco DM degradability determinations were conducted on d 8 and d 9 in each experimental period to determine the extent of rumen digestion of each of the three carbohydrate sources offered (rolled barley, maize meal, and soya hulls) over a 48 h period. *In situ* filter bags (5 × 10 cm; 50 µm pore size) (Ankom Technology, Macedon, New York, USA) containing approximately 5 g DM feed were placed inside large mesh nylon bags and inserted into the ventral sac of the rumen and secured with a metal weight. The *in situ* bags were inserted at 1700 h on d 8 of each experimental period and incubated for 0, 2, 4, 6, 8, 12, 24, 48 h in reverse order. All feed samples were previously ground using a Norris hammer mill fitted with a 2 mm screen (Lab Mill Christy Turner, Suffolk, UK). After removal from the rumen, all bags were immediately submerged in ice-cold water, thoroughly washed and frozen at -20 °C. Upon thawing, *in situ* bags were rinsed in a domestic washing machine for 30 min using the cold rinse cycle in the absence of detergent, then dried at 55 °C for 48 h. Degradability constants *a, b,* and *c* were estimated according to the non-linear model: $P = a + b\ (1\text{-e}^{-ct})$ (Ørskov and McDonald, 1979) where '*a*' represents the soluble degradable fraction , '*b*' represents the slowly degraded fraction within the rumen and '*c*' is the constant rate of degradation per hour of the '*b*' fraction with time '*t*'. Effective degradability (ED) was calculated using the equation $a + \underline{bc}\ /\ (c+k)$ (Ørskov

and McDonald, 1979), were *k* is the fixed rumen outflow rate 0.03 h^{-1} (Mulligan et al., 2002).

Rumen fluid samples were collected on d 10 and 11 while in the metabolism house via the cannula for pH, NH_3 and volatile fatty acids (VFA) determination. Samples were collected at 1, 2, 4, 6, 8 h post feeding for a total of 48 h. Rumen fluid was collected using a collection tube (#RT, Bar Diamond, Parma, ID) and 60 ml disposable syringe. At each time point 50 ml from five different sites within the rumen (anterior dorsal, anterior ventral, medial ventral, posterior dorsal, and posterior ventral sac of the rumen) was collected via cannula and pooled from each animal. The pH was immediately measured (Orion 3 Star pH Benchtop Meter, Thermo Scientific, Massachusetts, USA) and a 4 ml subsample taken using an automatic pipette and mixed with 1 ml TCA (500 g L^{-1} trichloroacetic acid) prior to storage at -20 °C for subsequent VFA and NH_3 analysis.

3.3.3 Chemical analysis

Samples of TMR, concentrates, GS and faeces were dried at 55 °C for 48 h in a forced air oven, ground in a hammer mill fitted with a 2 mm screen (Lab Mill, Christy Turner, Suffolk, UK) and stored for DM determination. The DM content of samples was determined after drying overnight at 105 °C (minimum 16 h) (method 930.15; AOAC, 1990). Ash concentrations were determined by complete combustion in a muffle furnace (Nabertherm Gmbh, Lilienthal, DE) at 550 °C for 5.5 h (method 942.05; AOAC, 1990). Starch was determined using the Megazyme Total Starch Assay Procedure (method 920.87; AOAC, 1990) (Product no: K-TSTA; Megazyme International Ireland LTD, Wicklow, IE (McCleary et al., 1994)). The N content of the feed and faeces samples were determined using the micro-Kjeldahl technique (method 920.87; AOAC, 1990). Samples were weighed and placed into flasks for

block digestion (unit model no. 435, Buchi, Postfach, Switzerland). Once digested, the samples were distilled (model no. 323, Buchi) into 50 ml boric acid (20 g L^{-1}) containing Tashiros indicator before titration. The N content of the urine was determined using a LECO FP 528 instrument (Leco Corp, St. Joseph, Michigan, US) (method 990.03; AOAC, 1990).

The apparent digestibility (%) of nutrients [DM, organic matter (OM), crude protein (CP), NDF and starch] was calculated according to the following equation (intake and output of nutrients in kilograms):

Apparent nutrient digestibility = (1 – (faecal nutrient/total nutrient intake)) × 100.

Neutral detergent fibre and acid detergent fibre (ADF) were determined by the method of Van Soest et al. (Van Soest et al., 1991) adopted for the use in the ANKOM ^{TM} 220 Fibre Analyser (ANKOM ^{TM} Technology, New York, US). Concentrate samples were analysed with a thermos-stable α-amylase and 20 g of Sodium sulphite ($NaSO_3$) was added to neutral detergent solution (NDS), while GS and faeces samples were analysed with NDS only. Neutral detergent fibre and ADF are expressed inclusive of residual ash. Gross energy of feed and faeces samples were determined by bomb calorimetry (Parr 1281 Bomb Calorimeter, Parr Instrument Company, Illinois, US). Ether extract was determined using Soxtex instruments (Tecator, Hoganas, Sweden) and light petroleum ether in feed samples only (method 920.85; AOAC, 1990).

Rumen fluid samples were thawed for 16 h at 4 °C and centrifuged at 1,800 × *g* for 10 min at 4 °C. One ml of supernatant was drawn off and diluted 1 in 5 with distilled water (dH_2O) and centrifuged at 1,800 × *g* for 15 min at 4 °C. From this, 200 µl supernatant was drawn off and NH_3 concentrations were determined using the phenol hypochlorite method of Weatherburn, (1967). For VFA analysis, a further sample containing 250 µl of supernatant was drawn off into a separate test tube and diluted

with 3.75 ml of dH_2O and 1 ml of internal standard (0.5 g 3-metyl-*n*-valeric acid in one litre of 0.15 M oxalic acid). Following centrifuging for five minutes, 260 × *g* at 21 °C, a sample was filtered through a 0.45 micrometre (μm) filter (Cronus Syringe filter PTFE 13 mm; SMI-LabHut Ltd., Maisemore, Gloucester, UK) into a 4 ml GC vial (Thermo Scientific, Langerwehe, Germany) and frozen at -20 °C until VFA analysis. One µl of sample was injected via an auto sampler on a Varian gas chromatograph (GC) 3800 with a 25 m X 0.53 mm i.d. megabore column (coating CP-Wax 58 (FFAP) – CB (no. CP7614), (Varian, Middelburg, Netherlands). The initial injector temperature was 75 °C, rising immediately to 95 °C, temperature increased at a rate of 3 °C min^{-1} to 200 °C (held for 50 s). Nitrogen was used as a carrier gas. The pressure of the column was held at 2.3 psi and the column rate was 8.1ml min^{-1}.

Blood plasma samples were analysed for glucose, urea, creatinine, and total protein. Plasma urea was determined using the enzymatic kinetic method, (Kit no. RX SERIES UR 3825), creatinine using colorimetric method (Kit no. RX SERIES CR3814), total protein using biuret reagent (Kit no. RX SERIES TP 4001), glucose using the hexokinase method (Kit no. RX SERIES GL 3816). All test kits were sourced from Randox Laboratories Ltd. (Antrim, Northern Ireland). All blood analyses were carried out using a clinical blood analyser (RX imola analyser RX4900; Randox Laboratories Ltd).

Data were analysed as a replicated 3 × 3 Latin square design using the PROC MIXED procedure of Statistical Analysis Software (SAS v9.4, Inst. Inc., Cary NC, USA) (Cooper et al., 2002). Normal distribution and homogeneity of variance were analysed using the UNIVARIATE procedure. Animal within period was the experimental unit. Model consisted of animal, period, and dietary treatment. Animal within period was a random effect. Ruminal data collected at different times after feeding were analysed

using the PROC MIXED procedure for repeated measures. The model contained the same fixed effects as before, except that time after feeding and its interaction with the main effects were included. Effects were considered significant at $P < 0.05$. When significant differences were detected, difference among treatment means and treatment by time point interaction were tested using Tukey's multiple comparison test.

3.4 Results

The effect of carbohydrate source on nutrient intake, and total tract apparent digestibility of nutrients is presented in Table 3.2. There was no difference among dietary treatments for dry matter, OM, or CP intake ($P > 0.05$). Animals offered SH had a higher NDF and lower starch intake compared to animals offered RB and MM ($P < 0.001$) whereas animals offered MM had a higher starch intake compared to animals offered RB ($P < 0.05$).

Total tract apparent digestibility of DM, OM, and CP did not differ ($P > 0.05$) between dietary treatments, however total tract digestibility of CP tended to be higher for RB compared to those offered MM ($P < 0.10$). Neutral detergent fibre total tract digestibility was higher for animals offered SH ($P < 0.001$) with no difference between animals offered RB and MM ($P > 0.05$). Starch total tract digestibility was higher for animals offered RB compared to animals offered MM ($P < 0.05$).

The *in sacco* ruminal digestion kinetics and effective degradability of carbohydrates is presented in Table 3.3. Fraction *a* (rapidly degradable component) was different between all three carbohydrate sources ($P < 0.001$) with rolled barley 10.0% higher compared to maize meal whereas soya hulls was 73.0% lower compared to rolled barley. The slowly degradable component *b* was 77.0% and 65.0% higher for soya

hulls compared to rolled barley and maize meal respectively ($P < 0.001$) while there was no difference between rolled barley and maize meal ($P > 0.05$). The factional rate of degradation per h of fraction *b* and *c*, was higher for rolled barley ($P < 0.01$) compared to maize meal and soya hulls which did not differ ($P > 0.05$). Effective degradability was lower for soya hulls compared to rolled barley and maize meal ($P < 0.001$), which did not differ ($P > 0.05$).

Table 3.2 The effect of concentrate carbohydrate source on nutrient intake and total tract apparent digestibility in beef heifers fed grass silage based diets.

	Dietary Treatment[1]				
	RB	MM	SH	SEM[3]	*P-value*
Intake (kg d $^{-1}$)					
Dry matter	6.04	6.03	6.03	0.031	0.958
Organic matter	5.65	5.62	5.60	0.028	0.442
Crude protein	0.89	0.87	0.87	0.011	0.577
Neutral detergent fibre	1.99[a]	1.89[b]	3.13[c]	0.009	0.001
Starch	1.12[a]	1.25[b]	0.04[c]	0.006	0.001
Apparent total tract digestibility, %					
Dry matter	76.24	74.90	75.03	0.635	0.311
Organic matter	77.74	76.33	76.62	0.643	0.310
Crude protein	72.02	66.82	68.12	1.396	0.061
Neutral detergent fibre	61.44[a]	59.89[a]	73.81[b]	0.831	0.001
Starch[2]	96.89[a]	95.67[b]	-	0.328	0.039

[abc] Within a row, means with a different superscript differ ($P < 0.05$)
[1]Grass silage based diet supplemented with rolled barley (RB), maize meal (MM) or soya hulls (SH)
[2]Total tract apparent starch digestibility RB vs MM
[3]SEM standard error of mean

Table 3.3 *In sacco* ruminal digestion kinetics[1] and effective degradability (ED) of carbohydrate sources fed to beef heifers on a grass silage based diet.

DM[2]	Rolled Barley	Maize Meal	Soya Hulls	SEM[3]	P value
a	0.641[a]	0.572[b]	0.170[c]	0.0045	<0.001
b	0.246[a]	0.381[a]	1.106[b]	0.0142	<0.001
b	0.371[a]	0.100[bc]	0.014[c]	0.0383	0.001
ED	0.877[a]	0.847[a]	0.568[b]	0.0181	<0.001

[abc] Within a row, means with a different superscript letter differ ($P < 0.05$)
[1]kinetics of digestions were estimated using the equation: $P = a + b\ (1\text{-e-}^{ct})$, a = soluble fraction; b = slowly degradable fraction; c = fractional rate of degradation per hour of the 'b' fraction with time 't'
ED calculated using the equation $a + \underline{bc}\ /\ (c+k)$, $k = 0.03\ h^{-1}$
[2]Dry matter disappearance
[3]SEM standard error of mean

The effect of carbohydrate type on N balance and blood metabolites is presented in Table 3.4. Nitrogen intake (g d^{-1}) was not affected by dietary treatment ($P > 0.05$). In addition, dietary treatment had no effect on total N excretion (g d^{-1}), the amount of N retained (g d^{-1}), and the amount of N excreted in the urine (g d^{-1}) ($P > 0.05$). There was a higher percentage of N excreted in the faeces and a lower percentage of N excreted in the urine when animals were offered MM compared to those offered RB ($P < 0.05$).

Blood plasma urea concentrations were higher for animals offered RB ($P < 0.01$), while no differences were observed for plasma creatinine and blood glucose levels between treatments ($P > 0.05$).

Table 3.5 shows the effect of carbohydrate source on rumen fermentation parameters. Animals offered SH had a higher ruminal pH than animals offered RB and MM ($P < 0.001$). Postprandial evolution of ruminal pH did not differ with dietary treatment ($P > 0.05$). Independent of dietary treatment, ruminal pH decreased, reaching nadir 1 h post feeding, and then gradually increasing to 6 h post feeding ($P < 0.001$).

There was a treatment × time interaction for rumen NH_3 concentrations ($P < 0.01$). At 1 h post feeding, animals offered the MM had higher rumen NH_3 concentrations than those offered the RB ($P < 0.05$), but this response was reversed at 4 and 6 h post feeding ($P < 0.01$), while at 6 h post feeding, NH_3 concentrations for the animals offered RB were higher than those offered the SH ($P < 0.05$). There were no differences observed between dietary treatments for ruminal NH_3 concentrations ($P > 0.05$). The animals offered RB had a higher concentration of ruminal valeric acid than those offered SH and MM ($P < 0.05$). No differences were observed between dietary treatments for ruminal; acetic acid, propionic acid, butyric acid, branched chain fatty acids (Iso valeric acid and Iso butyric acid) and total rumen VFA concentrations ($P >$

0.05). However, ruminal acetic acid concentrations tended to be higher for animals offered SH compared to MM ($P < 0.10$), and animals offered RB tended to have higher ruminal propionic acid and butyric acid concentrations compared to animals offered SH ($P < 0.10$). Animals offered MM had a higher ratio of A: P compared to animals offered SH ($P < 0.05$). Concentrations of ruminal; propionic acid, butyric acid, valeric acid, branched chain fatty acids (Iso valeric acid and Iso butyric acid) and total VFA concentrations ($P < 0.001$) were highest 2 h after feeding.

Table 3.4 The effect of concentrate carbohydrate source on nitrogen balance and blood metabolites in beef heifers fed grass silage based diets.

	Dietary Treatment[1]				
	RB	MM	SH	SEM[6]	*P*-value
N Intake (g d $^{-1}$)	143	142	143	4.0	0.105
N output (g d $^{-1}$)					
Urine N	81	76	82	4.0	0.553
Faecal N	39^{a}	46^{b}	43^{ab}	1.3	0.025
Total excretion	121	118	126	4.8	0.514
Retained	21.0	23.8	15.8	4.9	0.538
N recovery[2]					
Urine	0.57	0.51	0.57	0.031	0.250
Faeces	0.28	0.32	0.31	0.012	0.062
N excreted (%)[3]	85.19	83.21	85.21	2.360	0.777
NUE (%)[4]	14.80	16.79	14.79	2.360	0.777
% total excreted [5]					
Urine	67.62^{a}	61.23^{b}	65.17^{ab}	1.553	0.045
Faeces	32.37^{a}	38.77^{b}	34.82^{ab}	1.553	0.045
Urine metabolites					
Creatinine (µmol L^{-1})	183.4	215.2	180.4	55.99	0.882
Urea (mmol L^{-1})	5.72	6.13	8.46	1.30	0.352
Blood metabolites					
Urea (mmol L^{-1})	3.05^{a}	2.52^{b}	2.86^{b}	0.075	0.002
Creatinine (µmol L^{-1})	140.9	141.7	137.3	5.13	0.285
Glucose (mmol L^{-1})	3.76	3.77	3.83	0.050	0.616

^{abc} Within a row, means with a different superscript letter differ ($P < 0.05$)

[1]Grass silage based diet supplemented with rolled barley (RB), maize meal (MM) or soya hulls (SH)

[2]N recovery = N out [faeces, urine (g/d)]/N intake (g/d)

[3]N excreted = [faeces + urine output (g/d)]/ N intake (g/d) *100

[4]NUE nitrogen use efficiency

[5] % total excreted = [urine, faeces output (g/d)/Total N output (g/d) *100

[6]SEM standard error of mean

Table 3.5 The effect of concentrate carbohydrate source on rumen fermentation parameters in beef heifers fed grass silage based diets.

	Dietary treatment[1]					Time after feeding							
	RB	MM	SH	SEM[4]	Diet	0h	1h	2h	4h	6h	SEM[4]	Time	Diet x Time
pH	6.50^{a}	6.46^{a}	6.65^{b}	0.040	<0.01	6.73^{a}	6.40^{b}	6.43^{b}	6.53^{cd}	6.60^{d}	0.037	<0.001	0.110
(mmol L $^{-1}$)													
NH_3	2.80	2.70	2.61	0.133	0.53	1.95^{a}	2.98^{b}	4.03^{cd}	3.00^{bd}	1.56^{e}	0.125	<0.001	0.019
Acetic	66.53	66.33$^{\downarrow}$	68.97$^{\downarrow}$	0.801	0.080	66.82	66.86	68.43	66.94	67.36	0.982	0.747	0.153
Propionic	10.51$^{\downarrow}$	9.94	9.68$^{\downarrow}$	0.255	0.079	8.13^{a}	11.36^{b}	11.88^{cb}	9.86^{d}	8.98^{ad}	0.286	<0.001	0.772
Butyric	8.87$^{\downarrow}$	8.27	7.59$^{\downarrow}$	0.435	0.090	7.11^{a}	8.45^{b}	9.31^{c}	8.47^{bd}	7.89^{ab}	0.385	<0.001	0.584
Valeric	0.96^{b}	0.88^{a}	0.86^{a}	0.018	0.006	0.70^{a}	0.88^{be}	1.11^{c}	0.98^{d}	0.84^{e}	0.024	<0.001	0.304
Iso valeric	1.40	1.36	1.44	0.067	0.733	1.29^{ad}	1.38^{acd}	1.63^{b}	1.42^{c}	1.29^{d}	0.047	<0.001	0.372
Iso butyric	1.36	1.36	1.37	0.050	0.983	1.37^{a}	1.21^{b}	1.54^{c}	1.41^{ac}	1.30^{ab}	0.046	<0.001	0.447
A: P[2]	6.53^{a}	7.07^{b}	7.49^{b}	0.145	0.003	8.40^{a}	6.13^{bc}	5.90^{c}	6.99^{d}	7.74^{e}	0.173	<0.001	0.507
TVFA[3]	89.66	88.15	90.05	1.319	0.508	85.48^{ac}	90.18^{ab}	93.93^{ab}	89.15^{ac}	87.70^{c}	0.173	<0.001	0.345

^{abc} Within a row, means with a different superscript letter differ ($P < 0.05$); $^{\downarrow}$ tendency towards significant ($P < 0.10$)

[1] Grass silage based diet supplemented with rolled barley (RB), maize meal (MM) or soya hulls (SH)

[2] A: P = ratio of acetic acid to propionic acid (acetic ÷ propionic)

[3] TVFA = total volatile fatty acids

[4] SEM standard error of mean

3.5 Discussion

The hypothesis that offering a carbohydrate source that is rapidly degraded within the rumen would capture more N within the rumen and in turn reduce N excretion was rejected.

3.5.1 In sacco degradability

The results obtained in this study from the *in sacco* degradability of the three feed ingredients fed reveal the difference in ruminal DM degradation of each carbohydrate source. In cereal grains, starch generally represents a large proportion of the feed DM, with a positive correlation between the ED of DM and ED of starch (Offner et al., 2003). The high values obtained for fraction '*a*' (the rapidly degradable component) with rolled barley and maize meal indicate that most of the starch was immediately washed out upon immersion of the bags within the rumen. However, the high soluble rate obtained with these ingredients may have been over estimated due to mechanical particle loss (Nocek, 1988) or the smaller particle size of barley and maize compared to that of soya hulls (Jane et al., 1994; Pérez and Bertoft, 2010; Yang et al., 2014). The animals used herein were fed at maintenance and to account for the underestimation in the digestibility of nutrients due to higher rumen turnover rates, the rumen outflow rate was fixed at 0.03 h^{-1} (Mulligan et al., 2001).

3.5.2 N-balance

In the current study, N recovered in the urine was similar across all treatments at 55.0% of ingested N, whereas N recovered in the faeces tended to be higher for animals offered MM compared to those offered RB (32.0% vs. 28.0% respectively). The partitioning of N excreted into urine and faeces is largely dependent on diet, with up to 75% of N excreted in urine when high protein, high concentrates diets are fed (Cole et al., 2005; Hristov et al., 2011a; Swanson et al., 2003), but can be reduced to 52.0%

excreted N in urine when diets are formulated to NRC recommended CP concentrations (Waldrip et al., 2013). Similarly, Colmenero and Broderick, (2006) observed that dairy cows fed increasing levels of CP and RDP had higher ruminal NH_3 concentrations, resulting in higher levels of N excreted in the urine.

Urinary N excretion is an environmental concern, as it is a major contributor to NH_3 emissions because urea in the urine is rapidly hydrolysed to NH_3 due to the prevalence of urease in the faeces (Powell et al., 2011). Ammonia is the principal source of urea that is produced in the rumen from RDP fed to excess or an insufficient energy supply to rumen microbes, metabolised to urea in the liver and excreted in the urine (Colmenero and Broderick, 2006). It was hypothesized in the current study that offering rolled barley, which has a more rapid rate of ruminal fermentation than maize meal and soya hulls would capture more NH_3 within the rumen and lead to a reduction in urinary N excretion. However, urine excretion was unaffected by carbohydrate source and was the major route of N excretion across all dietary treatments (79 g d^{-1}). Ferreira et al. (2011a) observed that replacing maize corn with increasing levels of SH in the diets of lambs, increased urinary excretion. This increase in urinary excretion can be explained by the increase in DMI intake as the level of SH in the diet increased, while simultaneously increasing the intake of CP in the diet. Similarly, Yan et al. (2007), established that the correlation between N intake and DMI to be positive. Whereas, in this study feed intake was restricted to maintenance, to ensure that DMI had no influence on N intake due to difference in energy density between the three feed ingredients (Ferreira et al., 2011a), in addition to diets formulated to be isonitrogenous (142.6 g d^{-1}). The intake level of carbohydrates in the diet can impact the level of N excreted in the urine, as the rate and extent of carbohydrate fermentation

within the rumen determines the utilization of ruminal NH_3 for microbial synthesis (Hristov et al., 1997) and the type of protein therein (Castillo et al., 2000).

Offering maize meal, which is more resistant to rumen degradation, compared to rolled barley (Offner et al., 2003), increases the percentage of total N excreted in the faeces 39.3 vs 32.8 % respectively. Surber and Bowman (1998) reported similar findings with beef cattle offered maize meal, where degradation of maize starch within the rumen was lower than those offered rolled barley leading to higher levels of N excreted in the faeces (35 vs 30 g d^{-1}). The site and the extent of carbohydrate fermentation can influence the level of faecal N excretion. Faecal N is primarily of microbial origin with lesser amounts of undegraded feed protein and endogenous secretions (NRC, 1985). Despite no differences in urinary N excretion observed between treatments in this study (79 g d^{-1}), the animals offered MM excreted a higher amount of N in the faeces compared to animals offered RB. Despite the higher level of starch intake with the animals offered MM, the animals offered RB had a higher apparent total tract digestibility of starch in addition to a tendency for a higher apparent total tract digestibility of CP, which would suggest undigested protein in the starch/protein matrix with animals offered MM (Philippeau et al., 1999). Maize starch is more resistant to rumen degradation than other cereal grains, as the starch granules in maize are embedded in the protein matrix, prolamins, which are more resistant to degradation at higher pH (Hoffman et al., 2011).

3.5.3 Rumen pH

Rumen pH is a critical factor in the normal and stable function of the rumen because of its profound effect on microbial populations and fermentation products, and on physiological functions of the rumen, with typical ruminal pH in grain fed beef cattle ranging from 5.5 to 6.2 (Nagaraja and Titgemeyer, 2007). As the forage to concentrate

ratio of the diet is decreased with high dietary levels of rapidly fermentable carbohydrates such as starch with low levels of effective fibre the probability of acidosis increases. Low ruminal pH may have been anticipated in the current study with the high concentrate to forage ratio offered. However, as a result of animals being fed to maintenance, and for additional rumen fill, all diets were supplemented with 1 kg DM of barley straw. The provision of straw in the diet enhances the level of fibre and physically effective fibre in the rumen, promoting rumination and saliva secretion, helping to buffer the acids from the fermentation of the feed. In addition, higher pH values obtained in this current study may be associated with the decreased volume of rumen digesta (low DMI) and increased dilution rate of rumen liquid or because of the increased extent of chewing (Dado and Allen, 1995).

3.5.4 Rumen NH_3 concentration

There was no difference in mean ruminal NH_3 concentration between treatments. The overall mean ruminal NH_3 concentration was 2.48 mmol L^{-1}, which was lower than those reported in (He et al., 2015) but similar with (Rotger et al., 2006). Kang-Meznarich and Broderick, (1980) reported 1.94 to 5 mmol L^{-1} to be the optimum level of ruminal NH_3 concentration adequate for microbial synthesis and fibre digestion, suggesting the levels of ruminal NH_3 produced in this study were adequate. However, there was a time by treatment interaction. The initial increase in ruminal NH_3 concentration with the animals offered MM is likely as a response to the lower availability of carbohydrate in the MM compared to the other dietary treatments (Hristov et al., 2004). In addition, the lower levels of NH_3 associated with RB and SH in the initial 3 h post feeding suggests that more energy was available to allow for better capture of NH_3 by the rumen microbes (Tamminga, 1979). Across all dietary treatments, the highest ruminal NH_3 concentration was detected 2 h after feeding as a

response to the rapid degradation of all sources of dietary protein similar to findings of Grigsby et al. (1992).

3.5.5 Volatile fatty acid concentrations

The concentrations of VFA within the rumen is the net result of substrate consumed by the animal and their absorption rate (Bannink et al., 2006), with the rate of absorption increasing as the ruminal pH decreases (Dijkstra et al., 1993). The total rumen VFA concentrations observed in this study (90.43 mmol L^{-1}) were lower compared to similar studies (148 mmol L^{-1}) involving beef cattle offered carbohydrates differing in rumen degradation rates (Rotger et al., 2006). However, these diets were offered *ad libitum*, whereas, in this current study DMI was restricted to maintenance. As the mean ruminal pH in this current study never dropped below 6.0, the lower rumen VFA concentrations were more likely as a result of lower rumen VFA production due to lower DMI consumed (Dado and Allen, 1995) rather than greater VFA absorption through the rumen epithelium (Valkeners et al., 2008). While not significant, the higher concentrations of acetic acid observed with the animals offered SH is a result of the higher proportion of NDF within the diet and higher total tract digestibility of NDF in animals offered SH (Dado and Allen, 1995) and as a consequence resulted in a higher Acetic acid: Propionic acid compared to the animals offered RB. The starch contained in barley is more fermentable within the rumen compared with maize starch (Offner et al., 2003). However, the similar VFA concentrations observed in this study may be as a result of the different levels of processing associated with each ingredient (McAllister et al., 1993) compared to the dry rolling of barley, the grinding of the maize grain was finely ground which produced large numbers of fine particles, increasing the surface area of the endosperm for utilisation by the rumen micro-organisms (Owens et al., 1986). Similar

observations were noted when substituting maize meal with rolled barley in beef cattle (Feng et al., 1995), and with dairy cows (Casper et al., 1999; Tothi et al., 2003) and substituting maize meal with soya hulls (Grigsby et al., 1993).

3.6 Conclusion

Offering a carbohydrate source that is rapidly degraded within the rumen such as rolled barley did not alter ruminal NH_3 concentrations, or reduce N excretion in beef heifers offered GS based diets fed maintenance. Similar ruminal NH_3 concentrations were observed across all treatments, highlighting that protein degradation exceeded carbohydrate fermentation 2 h post feeding. In conclusion, supplementing grass silage-based diets with concentrate carbohydrate sources with different fermentation profiles had no effect on N metabolism of beef heifers fed to maintenance. However, this approach is unlikely in practice as the animals were fed to maintenance on a diet that contained 60% concentrates.

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Chapter 4

The effects of chitosan differing in molecular weight, at three different inclusion levels on rumen fermentation parameters *in vitro* using artificial Rumen Simulation Technique (RUSITEC).

4.1 Abstract

The investigation into novel additives, which may mitigate ammonia (NH_3) emissions from the ruminant sector have never been more desired due to failure to meet targets set in the National Emission Ceilings Directive, 2016. It was hypothesized that inclusion of chitosan, low in molecular weight would inhibit ruminal NH_3 degradation, and in turn reduce ruminal NH_3 concentrations *in vitro.* The objective of this study was to evaluate the effects of chitosan differing in molecular weight, at three different inclusion levels on the manipulation of rumen fermentation *in vitro* using artificial RUSITEC in a factorial designed experiment. The factors were molecular weight and inclusion level. Dietary treatments differed in molecular weight as follows: C0 (control) zero chitosan; C1 chitosan molecular weight <5,000 kDa; C2 chitosan molecular weight 10,000 kDa; C3 chitosan molecular weight 20,000 kDa; C4 chitosan molecular weight 150,000 kDa. Diets were fed at three different chitosan feeding levels; 500 mg L^{-1}, 1,000 mg L^{-1} and 1,500 mg L^{-1}, replicated three times and distributed evenly among five (eight vessel) RUSITEC machines. The inclusion of chitosan negatively affected dry matter, crude protein, nitrogen, and neutral detergent fibre disappearance ($P < 0.05$) compared to C0. Irrespective of molecular weight, chitosan increased ruminal NH_3 concentrations ($P < 0.05$). The inclusion of C4 increased total gas production, methane (CH_4) volume, CH_4 concentrations, and CH_4 concentrations expressed per gram of organic matter digested compared to all other treatments ($P < 0.001$).

4.2 Introduction

The environmental impact of animal production systems, such as the ruminant sector has received a lot of attention due to their impact on the environment, as they excrete excess nitrogen (N) in manure and emit methane (CH_4) in large quantities. Nitrogen use efficiency in beef cattle is low, with 80-90% of N excreted in the urine and faeces (Cole and Todd, 2009). The N substrates in high quality forages (pasture and grass silage) are mainly soluble and and very quickly available and degraded within the rumen (Min et al., 2005), leading to excess levels of ammonia (NH_3) in the rumen which is absorbed from the rumen and excreted in urine (Patra and Saxena, 2011). Therefore, a reduction of protein degradation in the rumen will increase the quantity of protein digested in the small intestine and reduce urinary N excretion. To date, the effects of dietary compounds on rumen fermentation has focused mainly on essential oils (Cobellis et al., 2016), saponins (Wallace et al., 2002) and tannins (Patra and Saxena, 2011). Chitosan (N-acetyl-D-glucosamine polymer) is a natural biopolymer formed by alkaline deacetylation of chitin (Walsh et al., 2012). Chitin is the second most abundant organic compound on earth next to cellulose, is found in the cell wall of lower plants and the exoskeletons of some arthropods and crustaceans (Kong et al., 2010). Due to its biodegradability and non-toxic properties, chitin has received much attention for its diverse applications in medicine and food preservation due to its antimicrobial properties (Cuero, 1999; Shahidi et al., 1999; Jeon et al., 2002; Kong et al., 2010). Chitosan should not be considered a single compound but rather a series of compounds with differing levels of deacetylation and other physicochemical characteristics (Goiri et al., 2009a). There have been studies with monogastrics (i.e. swine and poultry) illustrating that chitosan can alter protein fermentation in the lower gastrointestinal tract (Han et al., 2013: O'Shea et al., 2011) and decrease *Escherichia*

coli numbers in the gut while increasing nutrient digestibility (Walsh et al., 2012). Its use in ruminant nutrition has not been extensively investigated and any studies that have been done to date report conflicting results with the inclusion of chitosan sometimes reducing dry matter digestibility and neutral detergent fibre digestibility (Goiri et al., 2009c). However, Goiri et al. (2010) reported that in sheep fed chitosan, ruminal fluid NH_3 concentrations were reduced, while Dias et al. (2017) found that chitosan positively affected dry matter (DM) intake, digestibility and increased propionic acid concentrations. A considerable proportion (50%) of the total NH_3 produced in the rumen is produced by a cohort of bacteria called hyper-ammonia-producing bacteria (HAP), representing about 1% of the rumen bacterial population (Russell et al., 1991). They are characterized as having a high deamination ability with a low ability to utilize carbohydrates, gram positive and highly sensitive to ionophores such as monensin (Eschenlauer et al., 2002). Inhibiting the activity of HAP bacteria can considerably decrease NH_3 production in rumen (Patra, 2012).

It is hypothesized that the inclusion of chitosan, low in molecular weight, will inhibit ruminal NH_3 degradation, and in turn reduce ruminal NH_3 concentrations *in vitro.*

Therefore, the objective of this study is to evaluate the effects of chitosan differing in molecular weight, at three different inclusion levels on the manipulation of rumen fermentation *in vitro* using artificial Rumen Simulation Technique (RUSITEC).

4.3 Materials and methods

4.3.1 Animals and experimental licencing

All animal procedures described in this experiment were approved by the Animal Research Ethics Committee (AREC) (approval number: AREC-E-17-14-Pierce) in

University College Dublin (UCD) and conducted under the European Directive 2010/63 EU and S.I. No. 543 of 2012. Each person who carried out procedures on experimental animals, during this experiment, were authorised to do so by means of individual authorisation from the Health Products Regulatory Authority (HPRA). This experiment was conducted at UCD Lyons Research Farm, Celbridge, Naas, Co. Kildare, Ireland. W23 ENY2 (53^0 17' 56" N, 6^0 32' 18" W).

4.3.2 Experimental procedure, rumen inoculum, diets

Rumen fluid and solids were collected from eight rumen-cannulated beef heifers (667±53 kg BW) fed a TMR diet consisting of 40: 60 grass silage (GS): concentrate on a DM basis.

The *in vitro* experiment consisted of two incubation periods using three RUSITEC systems with eight vessels/system (Sanshin Industrial Co. Ltd, Yokohana, Japan), to simulate the rumen environment. Each vessel had an effective volume of 800 ml and kept at 39° C under permanent vertical agitation as described by Czerkawski and Breckenridge (1977).

The *in vitro* basal diet consisted of 40: 60 forage: concentrate on a DM basis. The concentrate contained one of five different chitosan treatments differing in molecular weight: CO (control) zero chitosan; C1 chitosan molecular weight <5,000 kDa; C2 chitosan molecular weight 10,000 kDa; C3 chitosan molecular weight 20,000 kDa; C4 chitosan molecular weight 150,000 kDa and fed at three different feeding levels 500 mg L^{-1}, 1,000 mg L^{-1} and 1,500 mg L^{-1}, arranged in a 5 × 3 factorial design, replicated three times, and distributed evenly among five (eight vessel) RUSITEC machines.

Each incubation period consisted of 16 d, with the first 10 d for microbial adaptation and fermentation stabilisation and the last 6 d for sampling. Collection of rumen

inoculum (fluid and digesta) took place before feeding at 0900. Solid digesta and rumen inoculum were collected, with the inoculum strained through four layers of cheesecloth. Rumen inoculum from all animals was pooled, flushed with carbon dioxide (CO_2), and incubated at 39° C before being transferred to the RUSITEC vessels within 45 minutes of collection. Each vessel was inoculated with 450 mL of rumen inoculum and 350 mL of anaerobic artificial saliva (Mc Dougall, 1948). Dietary treatments were added to each vessel in nylon bags (100 μm pore size; 5 × 10 cm – concentrate; 10 × 20 cm – forage; ANKOM ^{TM} Technology, Macedon, NY, USA); 70 g of rumen solid digesta and incubated in each vessel for 1 d to provide solid associated bacteria, with a second bag containing 8 g DM GS and a third concentrate bag containing 12 g DM concentrate plus chitosan treatment. The concentrate feed component was ground through a 1 mm sieve and the forage component was chopped to 2-4 cm in length using a bowl chopper. After 24 h, each vessel was opened and two of the initial three bags removed – bag containing rumen digesta solids and the bag containing the concentrate plus treatment of interest – squeezed and washed in 50 ml of artificial saliva. The liquid fractions of the washings were returned to the vessels and two new nylon bags, containing 8 g DM GS and 12 g DM of concentrate plus chitosan treatment of interest were inserted into the fermentation vessels. On subsequent days, the nylon bag containing GS that had been in the vessel for 48 h was replaced with a new nylon bag containing GS, and the nylon bag containing the concentrate of interest, which had been in the vessel for 24 h was replaced as described above.

Artificial saliva was prepared daily and was continuously infused at a rate of 640 ml d^{-1} (dilution rate of 3.33 % h^{-1}) to prevent the wash out of rumen microbes, using a

multichannel peristaltic pump (Watson-Marlow 500 series, Cornwall, UK). The displaced effluent and fermentation gasses from each fermentation vessels were collected into effluent bottles and gas collection bags, respectively.

4.3.3 Sampling

Dry matter degradation, gas production, methane (CH_4) and outflow of fermentation products were measured on d 11, 12, 13 and 14. Overflow vessels were kept in a water bath maintained at 2^o C to stabilise fermentation products. Samples of outflow liquor (4 ml) were collected using an automatic pipette and mixed with 1 ml TCA (500 g L^{-1} w/v trichloroacetic acid) and stored at -20° C for subsequent for volatile fatty acid (VFA) and NH_3 analysis. Fermentation gases were collected in reusable polyethylene gas-tight bags fitted with one-way valves that were attached to each outflow vessel. Total gas production was measured using a DC-1 dry gas test meter (Sinagawa Corp.; Tokyo, Japan), and CH_4 percentage was analysed using a GC100 portable CH_4 reader (ADC Gas Analysis; Hoddeston, UK). Nylon bags were collected and rinsed with cold water. Feed residues in the nylon bags were washed in a domestic washing machine using the cold rinse cycle in the absence of detergent (30 min) to remove the bacteria attached loosely to the bags. The feed residue was then dried in a 55° C forced air oven for 48 h and then weighed. Feed dry matter digestibility was calculated as the amount of material that disappeared from the nylon bags after 24 h and 48 h of incubation, for concentrates and GS, respectively. Chemical composition of the dried incubation residues was determined to calculate digestibility of feed components.

4.3.4 Chemical analysis

Dried samples of GS, concentrates and feed residues were dried at 55^o C for 48 h in a forced air oven, ground in a hammer mill fitted with a 2 mm screen (Lab Mill, Christy

Turner, Suffolk, UK) and stored for DM determination. The DM content of samples was determined after drying overnight at 105° C (minimum 16 h) (method 930.15; AOAC International, 2005). Ash concentrations were determined by complete combustion in a muffle furnace (Nabertherm Gmbh, Lilienthal, DE) at 550° C for 5 h (AOAC International, 2005). The N content was determined using a LECO FP 528 instrument (Leco Corp, St. Joseph, Michigan, US) (AOAC International, 2005).

Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the method of Van Soest et al. (1991) adopted for the use in the ANKOM ™ 220 Fibre Analyser (ANKOM ™ Technology, NY, US). Concentrate samples were analysed with a thermos-stable α-amylase and 20 g of Sodium sulphite ($NaSO_3$) was added to neutral detergent solution (NDS), while silage and faeces samples were analysed with NDS only. Neutral detergent fibre and ADF are expressed inclusive of residual ash. Gross energy (GE) of feed were determined by bomb calorimetry (Parr 1281 Bomb Calorimeter, Parr Instrument Company, Illinois, US). Ether extract (EE) was determined using Soxtex instruments (Tecator, Hoganas, SE) and light petroleum ether.

Rumen fluid samples were thawed for 16 h at 4° C and centrifuged at 1,800 × *g* for 10 min at 4° C. One ml of supernatant was drawn off, diluted 1 in 5 with dH2O, and centrifuged at 1,800 × *g* for 15 min at 4 °C. From this, 200 µl supernatant was drawn off and NH_3 concentrations were determined using the phenolyhpochlorite method of Weatherburn (1967). For VFA analysis, a sample containing 250 µl of supernatant was drawn off into a separate test tube and diluted with 3.75 ml of dH_2O and 1 ml of internal standard (0.5 g 3-metyl-n-valeric acid in one litre of 0.15 M oxalic acid). Following centrifuging for five minutes, 260 × *g* for 21° C, a subsample was filtered

through a 0.45 micrometre (μm) filter (Cronus Syringe filter PTFE 13 mm; SMI-LabHut Ltd., Maisemore, Gloucester, UK) into a four ml GC vial (Thermo Scientific, Langerwehe, Germany) and frozen at -20° C until VFA analysis. One μl of sample was injected via an auto sampler on a Varian gas chromatograph (GC) 3800 with a 25 m × 0.53 mm i.d. megabore column (coating CP-Wax 58 (FFAP) – CB (no. CP7614), (Varian, Middelburg, Netherlands). The initial injector temperature was 75° C, rising immediately to 95° C, temperature then increased at a rate of 3° C min^{-1} until the temperature reached 200° C, and was then held for 50 seconds. Nitrogen was used as a carrier gas. The pressure of the column was held at 2.3 psi and the column rate was 8.1 ml min^{-1}

4.3.5 Statistical analysis

Data were analysed using the PROC MIXED procedure of Statistical Analysis Software (SAS v9.4, Inst. Inc., Cary NC, USA). Normal distribution and homogeneity of variance were analysed using the UNIVARIATE procedure. Data that were not normally distributed were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box-Cox transformation analysis using the TRANSREG procedure of SAS. Fixed effects in the model included chitosan inclusion and feeding level and their interaction. Vessel was included as a random effect. Where interactions were not significant, this term was excluded from the model. Statistically significant differences between least squares means were tested using the PDIFF command incorporating the Tukey test for pairwise comparison of treatment means. Repeated measures (day) was also included in the model. Statistical significance was assumed at a value of $P < 0.05$ and a tendency toward significance assumed at a value of $P > 0.05$ but < 0.10.

4.4 Results

The interaction between chitosan inclusion and feeding level was investigated for all parameters but was non-significant and was therefore removed from the analysis.

The effect of chitosan inclusion and feeding level on nutrient disappearance is presented in Table 4.1. Chitosan had a negative effect on DM disappearance ($P > 0.05$), whereas inclusion level had no effect ($P > 0.05$). Dry matter disappearance was 8 % lower in vessels that received C2 and C3 relative to CO. Organic dry matter disappearance was not affected by chitosan inclusion and feeding level ($P > 0.05$). However, N disappearance was affected by chitosan inclusion ($P < 0.001$) and the feeding level ($P < 0.05$). Vessels that received no chitosan had the highest percentage of N degraded, whereas vessels that received C4 had the lowest percentage of N disappearance. Furthermore, offering chitosan at the highest inclusion level (1,500 mg L^{-1}) reduced N disappearance compared to 500 and 1,000 mg L^{-1} ($P < 0.05$).

The effects on chitosan inclusion and feeding level on *in vitro* fermentation are presented in Table 4.2. The overflow pH was higher in the C1 inclusion compared to the C4 inclusion level ($P < 0.05$). At all levels of chitosan inclusion, the NH_3 levels were higher than the control ($P < 0.05$). Total VFA concentrations were unaffected by chitosan inclusion but acetic acid and iso-valeric acid concentrations were affected ($P < 0.05$). Acetic acid concentrations were lower in vessels that received C3 compared to vessels that received C1 ($P < 0.05$), while the inclusion of C2, C3, and C4 decreased iso-valeric concentrations compared to the CO treatment ($P < 0.001$). Iso-valeric acid production was lower at the highest feeding level (1500 mg L^{-1}) compared to the control.

The inclusion of C4 increased total gas production, CH_4 volume, CH_4 concentrations, and CH_4 concentrations expressed per gram of OM digested compared to all other treatments ($P < 0.001$).

Table 4.1 The effects of chitosan differing in molecular weight, at three different inclusion levels on nutrient digestibility *in vitro* using the rumen simulation technique (RUSITEC).

	Treatments[1]							Feeding level[2]				
	CO	C1	C2	C3	C4	SEM	*P*-value	500	1000	1500	SEM	*P*-value
Disappearance												
DM	70.65^{a}	67.52^{abc}	65.32^{bc}	64.67^{c}	65.99^{abc}	1.180	0.045	66.79	65.17	65.66	0.964	0.489
OM	65.80	63.29	61.28	61.16	61.16	1.210	0.136	61.86	61.36	61.94	1.040	0.912
N	71.04^{a}	68.15^{ab}	63.98^{bc}	63.34^{c}	56.12^{d}	1.308	<0.001	64.74^{a}	62.87^{ab}	61.08^{b}	0.939	0.037
NDF	36.23^{a}	30.74^{ab}	26.16^{b}	25.11^{b}	29.95^{ab}	1.916	0.009	30.66	26.62	26.69	1.434	0.095

^{a,b,c} Different superscript letter within a row indicates significance ($P < 0.05$).

[1] molecular weight: CO, control; C1 <5,000 kDa; C2 10,000 kDa; C3 20,000 kDa; C4 150,000 kDa

[2]Feeding level mg L^{-1}

Table 4.2 The effects of chitosan differing in molecular weight, at three different inclusion levels on ruminal fermentation parameters *in vitro* using the rumen simulation technique (RUSITEC).

	Treatments[1]							Feeding level[2]				
	CO	C1	C2	C3	C4	SEM	*P*-value	500	1000	1500	SEM	*P*-value
pH	6.69^{ab}	6.70^{a}	6.69^{ab}	6.67^{ab}	6.62^{b}	0.021	0.025	6.67	6.67	6.66	0.016	0.919
Concentration (mmol L $^{-1}$)												
NH_3	2.07^{a}	2.59^{b}	2.85^{b}	2.94^{b}	2.77^{b}	0.164	0.05	2.56	2.82	2.98	0.132	0.098
Acetic	51.82^{ab}	58.38^{a}	51.84^{ab}	49.96^{b}	51.92^{ab}	2.255	0.032	53.24	52.08	53.49	1.672	0.815
Propionic	17.29	18.18	16.45	15.03	14.10	1.400	0.188	15.84	15.41	16.44	0.684	0.558
Butyric	6.21	6.85	7.15	7.01	8.08	0.475	0.155	7.27	7.12	7.43	0.402	0.859
Valeric	4.23	4.43	4.76	5.29	5.73	0.561	0.255	4.90	5.05	5.09	0.523	0.271
Iso-butyric	0.61	0.63	0.60	0.61	0.63	0.017	0.599	0.62	0.61	0.62	0.012	0.350
Iso-valeric	7.06^{a}	6.96^{a}	5.66^{bcd}	5.57^{bc}	5.83^{bcd}	0.199	<0.001	6.28^{a}	5.97^{ab}	5.75^{b}	0.151	<0.001
Total VFA	87.22^{a}	96.00^{b}	85.56^{a}	84.41^{a}	86.13^{a}	3.272	0.032	88.45	86.24	89.07	2.479	0.696
Total gas (Ld^{-1})	1.22^{a}	1.12^{a}	1.18^{a}	1.12^{a}	1.41^{b}	0.043	<0.001	1.22	1.19	1.23	0.033	0.644
Methane (Ld^{-1})	0.063^{a}	0.068^{a}	0.079^{a}	0.077^{a}	0.11^{b}	0.005	<0.001	0.086	0.080	0.083	0.004	0.517
Methane (%)	5.19$^{\downarrow}$	6.00	6.80	7.01	7.34$^{\downarrow}$	0.473	0.068	6.90	6.76	6.71	0.423	0.945
Methane (mmol d^{-1})	2.61^{a}	2.78^{a}	3.21^{a}	3.24^{a}	4.39^{b}	0.276	<0.001	3.53	3.30	3.41	0.211	0.744
Methane (mmol g^{-1}DOM)	34.42^{a}	35.09^{a}	38.56^{a}	39.62^{a}	51.41^{b}	3.274	<0.001	42.80	39.86	41.01	2.466	0.699

^{a,b,c} Different superscript letter within a row indicates significance ($P < 0.05$).
[1] molecular weight: CO, control; C1 <5,000 kDa; C2 10,000 kDa; C3 20,000 kDa; C4 150,000 kDa
[2]Feeding level mg L^{-1}

4.5 Discussion

The hypothesis was that the inclusion of chitosan, low in molecular weight will inhibit ruminal degradation, thus reduce ruminal NH_3 concentrations *in vitro* was rejected. Chitin in its pure form is insoluble, but when treated with sodium hydroxide, chitin is deacetylated to chitosan, which increases it solubility and activity. Variations in the deacetylation process influences chitins degree of acetylation and molecular weight, subsequently affecting the bioactivity of the resultant chitosan. Given the divergence in the molecular weight in the chitosan used in this study, (<5,000 – 120,000 kDa) the results observed are quite variable and had very little effect on *in vitro* rumen fermentation.

4.5.1 The effect of chitosan inclusion on DM disappearance

Previously the inclusion of chitosan *in vivo* improved DM, CP and NDF digestibility (Araujo et al., 2015; Mingoti et al., 2016; Vendramini et al., 2016; de Pavia et al., 2017). However, in the current study, the inclusion of chitosan reduced DM, CP and NDF digestibility. Similar results were observed when chitosan was included *in vitro* (Goiri et al., 2009a; Goiri et al., 2009b; Goiri et al., 2009c; Goiri et al., 2010; Belanche et al., 2016a). These decreases in the disappearance of feed from the nylon bags after 48 h indicate that *in vitro* fermentation of the basal diet may be negatively affected by the addition of chitosan, probably as a result of a decrease in ruminal bacteria and/or in their activity, likely due to the antimicrobial action of chitosan against ruminal microbes (protozoa and fibrolytic bacteria) (Wencelova et al., 2014; Belanche et al., 2016a).

4.5.2 The effect of chitosan inclusion on rumen fermentation

A previous study by Thao et al. (2014) reported that chitosan could reduce the production of NH_3 by inhibiting the deamination of amino acids by microorganisms in the rumen. The NH_3 concentration in the rumen fluid is an important indicator of the rumen environmental parameters, reflecting the supply of microbial N in the rumen (Firkins et al., 2007). However, in the current study, the inclusion of chitosan differing in molecular weight increased NH_3 concentrations. A similar response was observed when chitosan was offered to beef steers on TMR diets (Araújo et al., 2015) and pasture (Dias et al., 2017) fed chitosan. The increased concentrations of ruminal NH_3 was likely due to an extra supply of NH_3 from the degradation of amine groups in chitosan and a lower uptake of NH_3 by the rumen microbes, rather than increased proteolysis (Belanche et al., 2016a).

It has been suggested that chitosan could act as a potential rumen moderator and boost propionic acid profiles (Seankamsorn et al., 2020). However, the addition of chitosan herein, irrespective of the molecular weight and inclusion level, had no impact on VFA concentrations, which is not in agreement with the observed decreases of feed disappearance. However, this result was in line with previous findings of Goiri et al. (2009b), where the addition of chitosan may have affected microbial growth and the efficiency of microbial synthesis, initiating increased VFA production in relation to the fermentable substrate (Hvelplund, 1991). Furthermore, as less energy is dissipated in CH_4 production, more should be retained in VFA (Wallace et al., 1981). When propionic acid is produced in the rumen, it competitively consumes 2 mol of H_2 (Belanche et al., 2016b), thus effectively inhibiting the formation of CH_4 (Kang et al., 2013; Belanche et al., 2016a).

The effects of chitosan inclusion on CH_4 emissions in the current study are contrary to observations in previous studies where chitosan inclusion reduced CH_4 emissions (Goiri et al., 2009a; Goiri et al., 2009b; Goiri et al., 2009c; Goiri et al., 2010; Belanche et al., 2016a). The inclusion of chitosan with lower molecular weight (C1, C2, and C3) had no effect, while the inclusion of C4 (higher molecular weight) increased CH_4 emissions. Reductions in CH_4 emissions in previous studies can be attributed to a decrease in OM disappearance. However, in the current study, no reductions were observed between treatments for OM disappearance, and when expressed as CH_4 mmol g DOM^{-1} there was no difference, except for vessels that received C4, which saw an increase. Previous studies involving chitosan saw a reduction in total VFA and acetic acid concentrations, with an increase in the propionic acid concentrations without effect on the butyric acid concentrations. These observations are similar to the pattern normally found with CH_4 inhibitors that directly inhibit methanogenic archaea or the metabolic pathways of CH_4 synthesis (Chalupa et al., 1980; Domescik and Martin, 1999). Moreover, apart from a reduction in acetic acid concentrations with vessels that received C3, chitosan inclusion had no effect on VFA concentrations.

Several hypotheses have been proposed as the mode of action for chitosan. The widely accepted theory is that the polycationic nature of chitosan, due to the positive charges of the protonated amino groups (NH_3^+), allows it to interact with the negatively charged outer membrane of numerous micro-organisms, causing extensive alterations to the cell surface, leading to leakage of intracellular substances, resulting in cell death (Ma et al., 2017). The outer peptidoglycan layer is more accessible in Gram-positive than in Gram-negative bacteria, to which most of the fibrolytic bacteria belong (Kong et al., 2010). Goiri et al. (2009b,c) reported reductions in OM digestibility *in vitro* with

chitosan inclusion, signifying activity towards cellulolytic bacteria, while Belanche et al. (2016b) observed a decrease in protozoal activity and rumen cellulolytic bacteria (Belanche et al., 2016a) responsible for the decrease in feed degradation and fermentation rate.

The antimicrobial activity for chitosan is pH dependent. Chitosan is only soluble in an acidic environment, and the molecule becomes polycationic as pH below the molecule's pKa (6.3-6.5) with a stronger inhibitory effect at lower pH (Helander et al., 2001; Lim and Hudson, 2004), weakening as the environmental pH increases (Kong et al., 2008). Chitosan loses it antimicrobial effect as the pH environment moves towards pH 7 due to the presence of a large majority of positively uncharged amino groups as well as poor solubility of chitosan (Aiedeh and Taha, 2001; Papineau et al., 1991; Sudarshan et al., 1992). The lack of an effect of chitosan used in this study may be attributed to the pH (6.66 ± 0.04) values observed in the vessels. Belanche et al. (2016a) demonstrated the antimicrobial properties chitosan, which caused a change in the structure of the bacterial community, shifting the fermentation pattern towards propionic acid production, with vessel pH values of 5.87.

4.6 Conclusion

The inclusion of chitosan had no effect on rumen fermentation parameters in this study possibly due to the lack of antimicrobial effect as a result of the high pH reported. The source of chitosan, which had the greatest effect, was of marine origin with a high molecular weight, compared to the chitosan derived from fungal sources with a lower molecular weight. However, the effect of chitosan low in molecular weight of marine origin on the manipulation of rumen fermentation is yet to be investigated. Further *in*

vitro and *in vivo* research is needed to determine if chitosan of marine origin at low molecular weight has any role modifying rumen fermentation parameters.

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Chapter 5

Effect of chitosan inclusion and dietary crude protein level on nutrient intake and digestibility, ruminal fermentation, and nitrogen excretion in beef heifers offered a grass silage based diet

Published in Animals, March 2021

5.1 Abstract

Nitrogen (N) use efficiency in beef cattle is low (10-20%), resulting in large amounts of N excreted into the environment. The objective of this study was to evaluate the effects of chitosan inclusion and dietary crude protein (CP) level on nutrient intake and digestibility, ruminal fermentation, and N excretion in beef heifers. Eight Belgian Blue × Holstein Friesian cross beef heifers (752 ± 52 kg BW) were used in a 4 × 4 Latin square with a 2 × 2 factorial design. Factors were dietary CP concentration-high CP, 16% (HP) or low CP, 12 % (LP) – and chitosan inclusion – 0 or 10 g kg dry matter^{-1} (DM) offered at 50: 50 forage to concentrate ratio on a dry matter (DM) basis. Apparent total tract digestibility of DM, organic matter (OM), and CP were reduced ($P < 0.001$) with chitosan inclusion, whereas offering the HP diets increased apparent total tract digestibility of CP ($P < 0.001$). Offering the HP diets increased urinary N excretion ($P < 0.001$), while chitosan inclusion increased N excretion in faeces ($P < 0.05$). Ruminal pH was increased with chitosan inclusion ($P < 0.01$). There was a CP × chitosan interaction for rumen ammonia (NH_3) concentrations ($P < 0.05$). Including chitosan in the HP diets increased ruminal NH_3 concentration while having no effect on the LP diets. Urinary N excretion was increased with increased levels of CP, but chitosan inclusion increased the quantity of N excreted in the faeces.

5.2 Introduction

Agriculture, especially the livestock sector, faces increasing pressure to reduce its impact on the environment (EU) 2016/2284 (Duffy, 2019). Nitrogen (N) excreted into the environment as a result of agricultural practices contribute towards nitrous oxide (N_2O) emissions, a major greenhouse gas; ammonia (NH_3), one of four transboundary gases; and the contamination of ground water through the leaching of nitrate (NO_3^-) (Environmental Protection Agency, 2016). During the period 2002 to 2016, significant increases in NH_3 emissions were observed over several of the world's major agricultural regions (Warner et al., 2017). Approximately 80% of global NH_3 emissions are a result of anthropogenic sources (animal and crop agriculture) (Aneja et al., 2020). In Ireland, agriculture is responsible for 99.1% of NH_3 emissions, with the application of animal manures accounting for 90% of the total figure (Duffy, 2020). Since the abolition of EU milk quotas in 2015, NH_3 emissions from agriculture have increased by 1.6% as a result of increased cattle numbers and urea application (Duffy, 2020). As part of the National Emissions Ceilings (NEC) Directive (2016/2284/EC) (EEA, 2016), Ireland is obliged to reduce its annual NH_3 emissions to 104 kt by 2020, while current figures are estimated at 119 kt per annum (Duffy, 2020).

Due to a number of different factors (Angelidis et al., 2019), N-use efficiency in beef cattle is low at 10-20% (Cole and Todd, 2009), resulting in large amounts of ingested N being excreted in urine and faeces, with urinary N accounting for 60-80% of total N excretion (Erickson and Klopfenstein, 2010). Urinary N is of the greatest concern, as the majority of N in urine is in the form of urea (Todd et al., 2015). The rate of transformation of urea in urine to NH_3 is much faster compared to N excreted in faecal matter, a process that requires the enzyme urease, which is in abundance in faecal

matter (Jarvis et al., 1989; Varel et al., 1999). The relationship between dietary crude protein (CP) or N intake and N excretion in the urine is far stronger (R^2 = 0.74) than faecal N (R^2 = 0.21) (Mulligan et al., 2004) and strategies that reduce CP in the diet are generally associated with a reduction in urinary N output (Cole et al., 2006; Vasconcelos et al., 2009; da Silva et al., 2016).

Recently, chitosan, due to its biodegradability, antimicrobial, and nontoxic properties, has received much attention for its diverse applications in medicine and food preservation (Kong et al., 2010; Mehdizadeh et al., 2020; Duran and Kahve, 2020). Chitosan (*N*-acetyl-D-glucosamine polymer) is a natural biopolymer formed from the deacetylation of chitin (Belanche et al., 2016b). Chitin is the second most abundant organic compound on earth next to cellulose, and it is found in the cell walls of lower plants and the exoskeletons of some arthropods and crustaceans (Dias et al., 2017).

The inclusion of chitosan in ruminant diets has been shown to alter ruminal fermentation, with chitosan shifting the fermentation pattern towards a more energy-efficient pathway when included in vitro (Goiri et al., 2009a, Goiri et al., 2009b, Belanche et al., 2016a). Goiri et al. (2010) and Dias et al. (2017) noted reductions in ruminal NH_3 concentrations with chitosan inclusion in sheep and beef cattle diets. Studies involving dairy cows and beef cattle observed that chitosan inclusion shifted the volatile fatty acid (VFA) production pattern from acetic acid to propionic acid, thereby decreasing the acetic to propionic acid ratio (Araújo et al., 2015, de Paiva et al., 2017). In addition to changes in ruminal fermentation, some authors noted chitosan increased apparent digestibility of dry matter (DM), CP, and neutral detergent fibre (NDF), while having no effect on nutrient intake (Araújo et al., 2015, de Paiva et al., 2017). However, Mingoti et al. (2016) and Dias et al. (2017) observed similar results in apparent digestibility of DM and CP, while simultaneously observing reductions in

DM, CP, and NDF intake. To date, the effects of chitosan inclusion on ruminal fermentation has focused mainly on total mixed ration (TMR) consisting of corn silage (Araújo et al., 2015; Del Valle et al., 2017; Mingoti et al., 2016; Vendramini et al., 2016; de Paiva et al., 2017), with no research investigating the effects of chitosan inclusion on ruminal fermentation in beef cattle fed grass silage.

It was hypothesised that offering chitosan to beef cattle will alter N metabolism within the rumen, increase CP digestibility in low CP diets, and in turn reduce N excretion.

The objective of this study was to evaluate the effects of chitosan inclusion with two levels of dietary CP on nutrient intake and digestibility, ruminal fermentation, and N excretion in beef heifers offered 50: 50 grass silage (GS) concentrate ratio.

5.3 Materials and Methods

All procedures described in this experiment were approved by the Animal Research Ethics Committee (AREC) at University College Dublin (UCD) and conducted under experimental license from the Health Products Regulatory Authority (HPRA) (approval number: AE18982/P121) under the European directive 2010/63/EU and S.I. No. 543 of 2012. Each person who carried out procedures on experimental animals during this experiment were authorised to do so by means of individual authorisation from the HPRA. This experiment was conducted at UCD Lyons Research Farm, Celbridge, Naas, Co. Kildare, Ireland, W23 ENY2 (53°17′56″ N, 6°32′18″ W).

5.3.1. Experimental design and dietary treatments

Eight Belgian Blue × Holstein Friesian cross beef heifers (*Bos taurus* strain) with an initial body weight of 752 ± 52 kg, surgically fitted with permanent ruminal cannula (100 mm i.d.) (Bar Diamond Inc., Parma, ID, USA) 575 d previously to facilitate sampling of rumen contents, were used in a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments (n = 8). Factors were dietary CP level, high (16%) CP (HP) versus low (13%) CP (LP), with or without chitosan inclusion (10 g kg^{-1} DM) offered at 50: 50 forage to concentrate ratio on a DM basis (Table 1). Diets were offered *ad libitum* to ensure daily refusal of 100 g kg^{-1}.

Chitosan was >90% degree of deacetylation, moisture 8.5%, pH 8.5, ash <1%, viscosity 40 mPa s^{-1} (A and Z Food Additives Co., Ltd., Zhejiang, China).

Diets and chitosan inclusion were offered once daily as a TMR at 0800 using a Calan Data Ranger (American Calan, Northwood, NH, USA). The GS used during this experiment consisted of predominantly perennial ryegrass (*Lolium perenne* L.). The crop was felled during the early booth stage of vegetation (growth stage 410; (Zadoks et al., 1974)), wilted for 16 h, harvested with a precision chop forage harvester (mean

particle length 50 mm), and ensiled under black polythene cover without the use of an additive. Prior to the commencement of the experiment, samples of silage were taken for forage analysis (Agri-Food and Biosciences Institute, AFBI-Hillsborough, Large Park, Hillsborough, Co. Down, NI) using NIRS (FOSS NIR systems 5000; FOSS UK, Warrington, Cheshire, UK).

Each experimental period consisted of a 14 d dietary adjustment period where the animals were fed their respective diets using a Calan Broadbent controlled feeding system (American Calan, Northwood, NH, USA), followed by a 10 d experimental period where the animals were housed in metabolism stalls (1.4 × 1.8 m). During this period in the metabolism stalls, animals were allocated the first 3 d for acclimatization, followed by a 5 d N-balance study and 2 d rumen sample collection. While in the metabolism house, animals was assigned to their own individual stall for the duration of the experiment with *ad libitum* access to water.

5.3.2 Data and sample collection

During the N-balance study, all animals were fitted with a specially constructed harness system to facilitate the separate collection of urine and faeces. This allowed the urine to flow through a plastic pipe into a plastic container, which contained 150 mL of 250 ml L^{-1} sulphuric acid to prevent microbial degradation and the loss of volatile N as NH_3. Total faeces were collected in trays behind each animal. Urine and faeces were weighed daily following morning feeding.

Samples of concentrates (HP and LP) were collected weekly, while GS and TMR samples were collected daily and later pooled per treatment and per animal for each experimental period.

Table 5.1 Ingredient composition and chemical composition of dietary treatments.

Protein Level [1]	**HP**		**LP**	
Chitosan Inclusion [2]	+	-	+	-
Ingredient composition, % DM				
Grass silage	50	50	50	50
Rolled barley	26.75	26.75	31.55	31.55
Protein mix [3]	20.50	20.50	5.7	5.7
Soyhulls	-	-	10	10
Molasses	1.5	1.5	1.5	1.5
Mineral mix [4]	1.25	1.25	1.25	1.25
Chemical composition, % DM				
Dry matter %	35.95	35.79	35.58	35.39
Crude protein	16.38	16.30	13.27	13.30
RDP [5]	11.10	11.10	9.25	9.25
RUP [5]	5.28	5.28	3.80	3.80
Neutral detergent fibre	36.77	36.03	39.03	37.54
Neutral detergent fibre *forage*	51.24	51.24	51.24	51.24
Acid detergent fibre	22.20	21.89	24.52	23.91
Starch	11.96	11.68	13.77	14.10
Ether extract	2.87	2.49	2.12	2.59
Gross energy, MJ/kg	16.65	16.62	16.44	16.27

[1]HP, high CP (16%); LP, low CP (13%)
[2] Chitosan inclusion 10 g kg DM^{-1}.
[3]Rapeseed meal + maize distillers grains 50:50.
[4]Vitamins/minerals consisted of the following: calcium carbonate, sodium chloride, mono-dicalcium, phosphate, and (sugar) beet molasses. Additives per kg: Vit A (retinol) (E672) 200,000 IU, Vit D_3 (cholecalciferol) (E671) 40,000 IU, Vit E (ali-rac-aipha-tocopheryl acetates) (3a700) 1000 IU. Compounds of trace elements: basic cobalt (II) carbonate monohydrate (3b302) 175 mg, copper sulphate pentahydrate 12,000 mg, ferric oxide (E1) 4790 mg, calcium iodate anhydrous (E2) 794 mg, manganous oxide (E5) 3226 mg, zinc oxide (E6) 5556 mg, sodium selenite (E8) 100 mg. Constituents: crude ash 96%, calcium 20%, phosphorus 2%, sodium 15%, copper added 3000 mg, selenium added 45 mg.
[5]Calculated using NRC (2001) model based on actual composition of feeds.

Samples were dried at 55 °C for 48 h for chemical analysis with additional samples frozen and stored at -20 °C for later total N analysis. Each morning during the N-balance study, faeces were mixed per animal and a 5% faecal sample collected. These samples were then split; a 10% sample was taken and frozen to -20 °C for subsequent chemical analysis, with the remaining sample dried at 55 °C for 240 h in a forced air oven. Dried and fresh samples of faeces were composited on an animal basis for each N-balance period. Urine samples were collected each morning with a 2.5% volume sample frozen to -20 °C.

On day 1 and 5 of each N-balance period, blood samples were collected by jugular venepuncture at 16:00 into blood collection tubes containing lithium heparin (REF: 367526, BD-Plymouth, UK), centrifuged at 1600× *g* for 20 min at 4 °C for plasma extraction. These samples were then stored at -20 °C pending analysis for plasma urea nitrogen, total protein, and creatinine concentrations.

Rumen fluid samples were collected on day 9 and 10 while in the metabolism house via the cannula for pH, NH_3, and VFA determination. Samples were collected at 0, 1, and 2 h, and then every subsequent 2 h post feeding for a total of 48 h. Rumen fluid was collected using a collection tube (#RT, Bar Diamond, Parma, ID, USA) and 60 mL disposable syringe. At each time point, 50 mL of rumen fluid from five different sites within the rumen (anterior dorsal, anterior ventral, medial ventral, posterior dorsal, and posterior ventral sac of the rumen) was collected via cannula and pooled for each animal. The pH was immediately measured (Orion 3 Star pH Benchtop Meter, Thermo Scientific, Waltham, MA, USA) and a 4 mL sample was collected using an automatic pipette and mixed with 1 mL TCA (500 g L^{-1} *w*/*v* trichloroacetic acid) prior to storage at -20 °C for subsequent VFA and NH_3 analysis.

5.3.3 Chemical analysis

Samples of TMR, concentrates, GS, and faeces were dried at 55 °C for 48 h in a forced air oven, ground in a hammer mill fitted with a 2 mm screen (Lab Mill, Christy Turner, Suffolk, UK), and stored for DM determination. The DM content of samples was determined after drying overnight at 105 °C (minimum 16 h) (method 930.15; AOAC ((AOAC). 1990)). Ash concentrations were determined by complete combustion in a muffle furnace (Nabertherm Gmbh, Lilienthal, Germany) at 550 °C for 5.5 h (method 942.05; AOAC ((AOAC). 1990)). Starch was determined using the Megazyme Total Starch Assay procedure (method 920.87; AOAC ((AOAC). 1990)) (product no: K-TSTA; Megazyme International Ireland LTD, Wicklow, Ireland; (McCleary et al., 1994)). The N content of the feed and faeces samples were determined using the micro-Kjeldahl technique (method 920.87; AOAC ((AOAC). 1990)). Samples were weighed and placed into flasks for block digestion (unit model no. 435, Buchi, Postfach, Switzerland). Once digested, the samples were distilled (model no. 323, Buchi) into 50 mL boric acid (20 g L^{-1}) containing Tashiros indicator before titration. The N content of the urine was determined using a LECO FP 528 instrument (Leco Corp., St. Joseph, MI, USA) (method 990.03; AOAC ((AOAC). 1990)). The apparent digestibility (%) of nutrients (DM, OM, CP, NDF, and starch) were calculated according to the following equation (intake and output of nutrients in kilograms):

Apparent nutrient digestibility = (1 − (faecal nutrient/total nutrient intake)) × 100

Neutral detergent fibre and acid detergent fibre (ADF) were determined by the method of Van Soest et al. (Van Soest et al., 1991), adopted for the use in the ANKOM^{TM} 220 Fibre Analyser (ANKOM^{TM} Technology, Macedon, NY, USA). Concentrate samples were analysed with a thermos-stable α-amylase and 20 g of sodium sulphite ($NaSO_3$)

was added to neutral detergent solution (NDS), while GS and faeces samples were analysed with NDS only. Neutral detergent fibre and ADF are expressed inclusive of residual ash. Gross energy of feed and faeces samples were determined by bomb calorimetry (Parr 1281 Bomb Calorimeter, Parr Instrument Company, Moline, IL, USA). Ether extract was determined using Soxtex instruments (Tecator, Hoganas, Sweden) and light petroleum ether in feed samples only (method 920.85; AOAC ((AOAC). 1990)).

Rumen fluid samples were thawed for 16 h at 4 °C and centrifuged at 1800 × *g* for 10 min at 4 °C. One ml of supernatant was drawn off, diluted 1 in 5 with distilled water (dH2O), and centrifuged at 1800 × *g* for 15 min at 4 °C. From this, 200 µL supernatant was drawn off and NH_3 concentrations were determined using the phenolyhpochlorite method of Weatherburn (Weatherburn, 1967). For VFA analysis, a further sample containing 250 µL of supernatant was drawn off into a separate test tube and diluted with 3.75 mL of dH_2O and 1 mL of internal standard (0.5 g 3-metyl-*n*-valeric acid in one litre of 0.15 M oxalic acid). Following centrifuging for 5 min, 260× *g* at 21 °C, a sample was filtered through a 0.45 micrometre (µm) filter (Cronus syringe filter PTFE 13 mm; SMI-LabHut Ltd., Maisemore, Gloucester, UK) into a four ml GC vial (Thermo Scientific, Langerwehe, Germany) and frozen at -20 °C until VFA analysis. One µL of sample was injected via an auto sampler on a Varian gas chromatograph (GC) 3800 with a 25 m × 0.53 mm i.d. megabore column (coating CP-Wax 58 (FFAP) − CB (no. CP7614), (Varian, Middelburg, The Netherlands). The initial injector temperature was 75 °C, rising immediately to 95 °C, temperature increased at a rate of 3 °C min^{-1} to 200 °C (held for 50 s). Nitrogen was used as a carrier gas. The pressure of the column was held at 2.3 psi and the column rate was 8.1 mL min^{-1}.

Blood plasma samples were analysed for glucose, urea, creatinine, and total protein. Plasma urea was determined using the enzymatic kinetic method, (kit no. RX SERIES UR 3825), creatinine using colorimetric method (kit no. RX SERIES CR3814), total protein using biuret reagent (kit no. RX SERIES TP 4001), glucose using the hexokinase method (kit no. RX SERIES GL 3816). All test kits were sourced from Randox Laboratories Ltd. (Antrim, Northern Ireland, UK). All blood analyses were carried out using a clinical blood analyser (RX Imola analyser RX4900; Randox Laboratories Ltd.).

5.3.4 Statistical analyses

Data were analysed using the PROC MIXED procedure of Statistical Analysis Software (SAS v9.4, Inst. Inc., Cary, NC, USA) for a 4 × 4 Latin square design with a 2 × 2 arrangement of treatments. Normal distribution and homogeneity of variance were analysed using the UNIVARIATE procedure. Data that were not normally distributed were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box–Cox transformation analysis using the TRANSREG procedure of SAS. The model accounted for chitosan inclusion and CP level; the interaction of CP level × chitosan inclusion as a fixed effect; and square, period within square and animal within square as random effect.

Ruminal data collected at different times after feeding were analysed using the PROC MIXED procedure for repeated measures. The model contained the same fixed effects as before, except that time after feeding and its interaction with the main effects were included.

Effects were considered significant at $p < 0.05$. When significant differences were detected, difference among treatment means and treatment by time point interaction were tested using Tukey's multiple comparison test.

For each variable, analysed data were subjected to the following covariate structures: unstructured, variance components, compound symmetric, and toeplitz. The covariance structure that yielded the smallest Schwarz's Bayesian criterion value was considered the most desirable for analysis.

5.4 Results

5.4.1 Nutrient intake and digestibility

The effect of CP level and chitosan inclusion on nutrient intake and digestibility are presented in Table 5.2. Crude protein level and chitosan inclusion had no effect on DMI ($P > 0.05$). Chitosan inclusion decreased the apparent total tract digestibility of DM, OM, and CP ($P < 0.01$), while having no effect on apparent total tract digestibility of NDF ($P > 0.05$). Animals offered HP diets had increased apparent total tract digestibility of CP ($P < 0.001$).

5.4.2 Nitrogen intake and output

Nitrogen intake ranged from 262.1 g d^{-1} to 325.8 g d^{-1} and was higher for animals offered HP diets ($P < 0.001$) versus those offered LP diets. Chitosan inclusion had no effect on N intake ($P > 0.05$). Animals that were offered HP diets excreted more N in urine compared to those offered LP diets ($P < 0.001$), with chitosan inclusion having no effect on urinary N excretion ($P > 0.05$). Furthermore, CP level had no effect on total N excreted in the faeces ($P > 0.05$), whereas including chitosan in the diet increased total N excreted in the faeces ($P < 0.05$). The animals that were offered HP diets excreted more total N ($P < 0.001$) compared to LP diets, while including chitosan in the diet had no impact on total N output ($P > 0.05$).

Feeding HP diets resulted in a higher proportion of N excreted in the urine ($P < 0.001$; 62.33 v 52.92%), while chitosan inclusion had no effect. Animals that were offered LP diets excreted a higher proportion of N in faeces compared to animals that were offered HP diets ($P < 0.001$), with no difference observed compared to the animals that were offered chitosan. Feeding HP diets increased the N recovered in the urine ($P < 0.01$), while higher proportions of N were recovered in the faeces with LP diets ($P < 0.001$) and chitosan inclusion ($P < 0.01$).

Table 5.2 Effect of chitosan inclusion and dietary crude protein level on dry matter intake, nutrient digestibility, nitrogen excretion, and blood metabolites in beef heifers offered grass silage based diet.

Crude Protein [1]	**HP**		**LP**		**SEM**	**CP**	**CHI**	**CP×CHI**
Chitosan [2]	+	-	+	-				
DMI (kg d^{-1})	12.31	13.14	12.14	12.33	0.373	0.206	0.183	0.395
Apparent total tract digestibility %								
Dry matter	67.50	69.95	67.22	69.29	0.818	0.427	<0.001	0.745
Organic matter	69.97	72.27	70.03	71.78	0.504	0.675	<0.001	0.589
Crude protein	68.83	71.47	61.36	64.38	0.009	<0.001	0.003	0.826
NDF[3]	48.00	50.59	49.08	50.59	1.313	0.688	0.133	0.689
Nitrogen								
Intake (g d^{-1})	323.8	327.9	262.3	261.9	0.009	<0.001	0.850	0.819
Output (g^{-1})								
Urine	166.1	159.8	106.1	113.8	0.009	<0.001	0.942	0.444
Faecal	100.4	93.5	101.9	93.8	0.003	0.797	0.041	0.865
Total	266.5	253.3	208.1	207.6	0.010	<0.001	0.502	0.534
% total excreted [4]								
% urine	62.15	62.51	51.09	54.76	1.790	<0.001	0.272	0.366
% faecal	37.85	37.49	48.91	45.24	1.790	<0.001	0.272	0.366

[1] HP, high CP (16%); LP, low CP (13%).
[2] Chitosan inclusion 10 g kg DM^{-1}.
[3] NDF, neutral detergent fibre.
[4]% total excreted = [urine, faeces output (g d^{-1})/Total N output (g d^{-1}) × 100.
[5] N recovery = N out [faeces, urine (g d^{-1})]/N intake (g d^{-1}).
[6] mmol L^{-1}.
[7] µmol L^{-1}.
[8] g L^{-1}

Table 5.2 (cont'd) Effect of chitosan inclusion and dietary crude protein level on dry matter intake, nutrient digestibility, nitrogen excretion, and blood metabolites in beef heifers offered grass silage based diet.

Crude Protein [1]	**HP**		**LP**		**SEM**	**CP**	**CHI**	**CP×CHI**
Chitosan [2]	+	-	+	-				
Nitrogen recovery[5]								
Urine	0.52	0.49	0.41	0.44	0.029	0.008	0.976	0.291
Faecal	0.31	0.28	0.39	0.36	0.009	<0.001	0.003	0.826
Blood metabolites								
Urea [6]	5.48	5.31	4.08	3.88	0.147	<0.001	0.209	0.933
Creatinine [7]	116.9	116.7	123.6	119.5	2.145	0.031	0.312	0.371
Total Protein[8]	81.45	81.63	79.91	81.84	1.419	0.636	0.462	0.534
Glucose [6]	3.60	3.56	3.61	3.57	0.078	0.911	0.611	0.956

[1] HP, high CP (16%); LP, low CP (13%).
[2] Chitosan inclusion 10 g kg DM^{-1}.
[3] NDF, neutral detergent fibre.
[4] % total excreted = [urine, faeces output (g d^{-1})/Total N output (g d^{-1}) × 100.
[5] N recovery = N out [faeces, urine (g d^{-1})]/N intake (g d^{-1}).
[6] mmol L^{-1}.
[7] µmol L^{-1}.
[8] g L^{-1}

5.4.3. Blood metabolites

Blood plasma urea was higher for animals offered HP diets ($P < 0.01$), while plasma creatinine was higher for animals offered LP diets ($P < 0.05$). No differences were observed for blood glucose levels and total protein between the two levels of CP offered ($P > 0.05$). Chitosan supplementation had no effect on any of the blood metabolites measured ($P > 0.05$).

5.4.4. Rumen fermentation parameters

The effect of chitosan inclusion and dietary crude protein level on rumen fermentation parameters are presented in Table 5.3. The level of CP that was offered had no effect on ruminal pH ($P > 0.05$), whereas animals that were offered chitosan had a higher ruminal pH ($P < 0.01$). Similar diurnal changes in pH were observed in all experimental diets ($P < 0.001$); with gradual decreases in ruminal pH observed until 14 h post feeding (Figure 5.1).

There was a CP × chitosan interaction for rumen NH_3 concentrations ($P < 0.01$). Animals offered HP with chitosan included had higher ruminal NH_3 concentrations, whereas chitosan had no impact on ruminal NH_3 concentrations in animals that were offered LP diets. There was a CP × time interaction for rumen NH_3 concentrations ($P < 0.01$). Ruminal NH_3 concentrations for animals offered HP diets peaked 2 h post feeding, while the concentrations for animals offered LP diets peaked 1 h post feeding (Figure 5.2).

Animals offered LP diets had higher concentrations of ruminal total VFA and acetic acid ($P < 0.001$), whereas those offered HP diets had higher concentrations of propionic acid ($P < 0.001$) (Figure 5.3). Crude protein level or chitosan inclusion had no effect on ruminal butyric acid concentrations ($P > 0.05$). The animals offered LP diets had a higher acetic to propionic acid ratio (A: P) compared to the animals offered

HP diets ($P < 0.001$), while including chitosan in the LP diets tended to decrease the A: P ($P < 0.10$). Crude protein level and chitosan inclusion had no effect on ruminal valeric acid, iso-valeric acid, and iso-butyric acid concentrations ($P > 0.05$).

Table 5.3. Effect of chitosan inclusion and dietary crude protein level on rumen fermentation parameters in beef heifers offered a grass silage based diet.

Crude Protein [1]	**HP**		**LP**		**SEM**	**CP**	**CHI**	**CP×CHI**
Chitosan [2]	+	-	+	-				
pH	6.47	6.44	6.45	6.43	0.025	0.163	0.002	0.870
Concentration (mmol L $^{-1}$)								
NH_3	2.69 [a]	2.45 [b]	1.75 [c,d]	1.78 [d]	0.084	<0.001	0.023	0.004
Acetic	111.2	110.7	115.3	117.3	1.62	<0.001	0.497	0.240
Propionic	14.26	14.36	13.70	13.44	0.234	<0.001	0.564	0.200
Butyric	9.24	9.33	9.00	9.25	0.206	0.338	0.355	0.633
Iso-butyric	1.87 [a]	1.75 [a]	1.68 [b]	1.86 [a,b]	0.113	0.490	0.792	0.052
Valeric	2.23	2.23	2.20	2.21	0.054	0.259	0.799	0.849

[a,b,c] Different superscript letter within a row indicates significance ($P < 0.05$)
[1] HP, high CP (16%); LP, low CP (13%).
[2] Chitosan inclusion 10 g kg DM^{-1}.
[3] VFA, volatile fatty acids.
[4] A: P = ratio of acetic acid to propionic acid (acetic ÷ propionic).

Table 5.3. (cont'd) Effect of chitosan inclusion and dietary crude protein level on rumen fermentation parameters in beef heifers offered a grass silage based diet.

Crude Protein [1]	**HP**		**LP**		**SEM**	**CP**	**CHI**	**CP×CHI**
Chitosan [2]	**+**	**-**	+	-				
Concentration (mmol L $^{-1}$*)*								
Iso-valeric	3.51	3.48	3.66	3.61	0.092	0.474	0.930	0.956
Total VFA[3]	142.2	141.8	145.6	147.1	1.98	<0.001	0.640	0.449
A: P [4]	8.51 [a]	7.80 [a]	9.07 [b]	9.58 [b]	0.586	<0.001	0.403	0.005

[a,b,c] Different superscript letter within a row indicates significance ($P < 0.05$)
[1] HP, high CP (16%); LP, low CP (13%).
[2] Chitosan inclusion 10 g kg DM^{-1}.
[3] VFA, volatile fatty acids.
[4] A: P = ratio of acetic acid to propionic acid (acetic ÷ propionic).

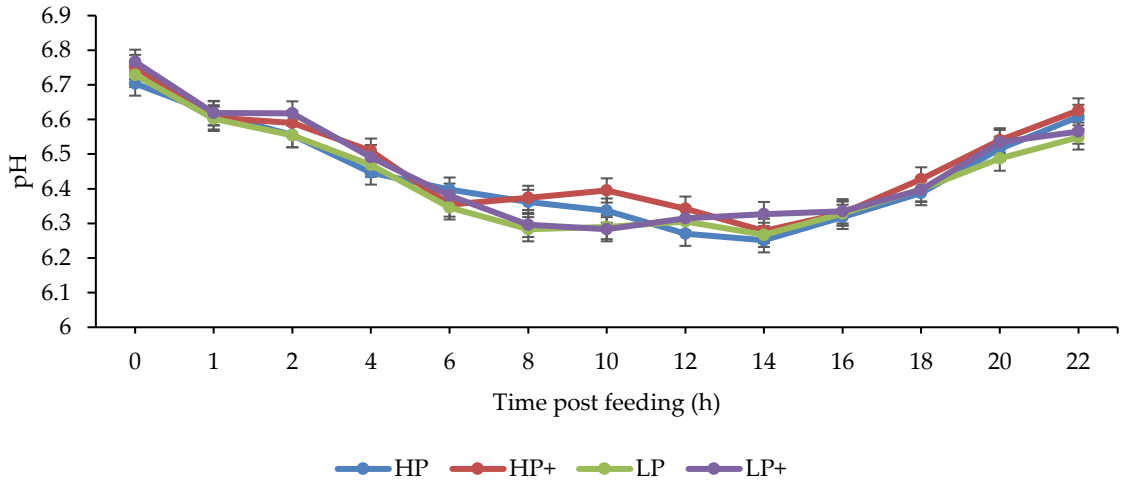

Figure 5.1. Effect of chitosan inclusion and level of crude protein on ruminal pH. Crude protein × chitosan $P > 0.05$; crude protein $P > 0.05$; chitosan $P < 0.01$; time after feeding $P < 0.001$.

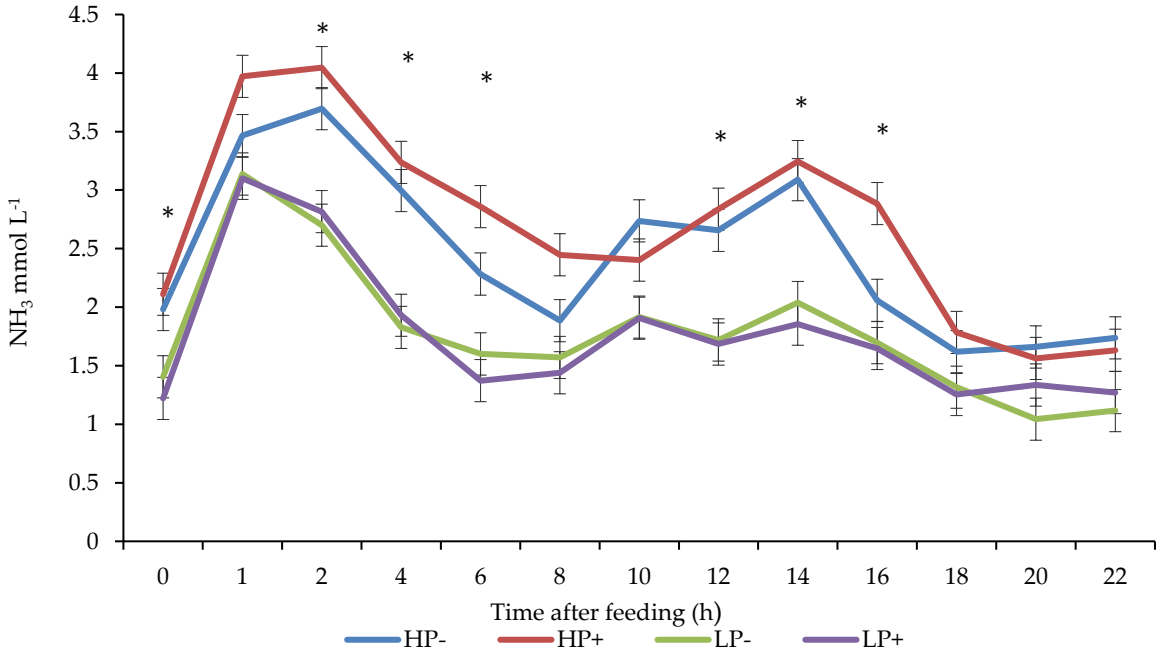

Figure 5.2 Effect of chitosan inclusion and level of crude protein on ruminal ammonia concentrations. * Denotes significance at time points between HP and LP. Crude protein × chitosan $P < 0.01$; crude protein $P < 0.01$; chitosan $P < 0.05$; time after feeding $P < 0.001$.

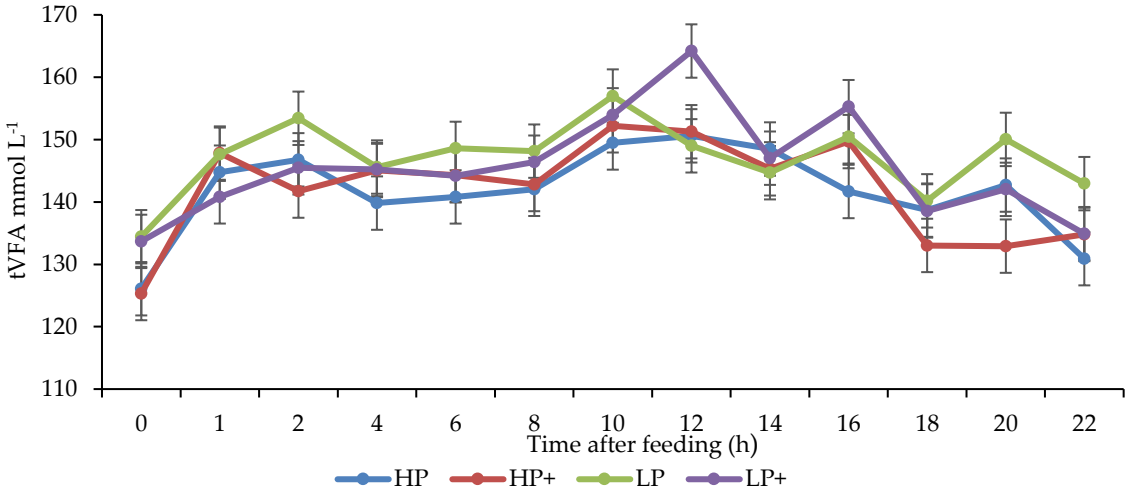

Figure 5.3 Effect of chitosan inclusion level and crude protein on ruminal total volatile fatty acid concentrations. Crude protein × chitosan $P > 0.05$; crude protein $P < 0.001$; chitosan $P > 0.05$; time after feeding $P < 0.001$.

5.5 Discussion

5.5.1. Nutrient intake and digestibility

The inclusion of chitosan in the diet reduced DM, OM, and CP digestibility; however this did not result in an alteration of DMI. This reduction in nutrient digestibility was likely due to the antimicrobial action of chitosan against ruminal microbes (protozoa and fibrolytic bacteria) (Belanche et al., 2016a; Wencelova et al., 2014). Protozoa play an important role in protein degradation in ruminants (Bach et al., 2005), with defaunation usually resulting in decreased protein degradation (Ivan, 2008). Several hypotheses have been proposed as the mode of action for chitosan. The widely accepted theory is the polycationic nature of chitosan, due to the positive charges of the protonated amino groups (NH_3^+), allows it to interact with the negatively charged outer membrane of numerous micro-organisms, causing extensive alterations to the cell surface, leading to leakage of intracellular substances, resulting in cell death (Ma et al., 2017). The outer peptidoglycan layer is more accessible in Gram-positive than in Gram-negative bacteria, of which most of the fibrolytic bacteria belong (Kong et al., 2010). Goiri et al. (2009a, 2009b) reported reductions in OM digestibility in vitro with chitosan inclusion, signifying activity towards cellulolytic bacteria, while Belanche et al. (2016b) observed a decrease in protozoal activity and rumen cellulolytic bacteria (Belanche et al., 2016a) responsible for the decrease in feed degradation and fermentation rate. Reducing the solubility of chitosan (<85% deacetylated) and its inclusion rate can diminish the negative impact on feed digestibility (Belanche et al., 2016b). In the current study, the average daily intake of chitosan was 10 g kg DM^{-1} with a degree of deacetylation >90%. Studies where no effect or increased nutrient digestibility was observed had an average daily intake of chitosan <7.75 g kg DM^{-1} with a degree of deacetylation ≤86% (Araújo et al., 2015;

de Paiva et al., 2017; Mingoti et al., 2016; Dias et al., 2017; Vendramini et al., 2016; Del Valle et al., 2017).

5.5.2. Nitrogen intake and output

There was no effect of feeding chitosan on N intake in this study. Similar results were observed by (Vendramini et al., 2016; Mingoti et al., 2016) when included in diets offered to lactating dairy cows, whereas in a dose response study (Dias et al., 2017), chitosan quadratically affected DMI as a result of a higher intake of CP and increases in nutrient digestibility. Intake of N not used by ruminal microorganisms in microbial protein synthesis is degraded to NH_3, metabolised to urea in the liver, and excreted in the urine. The manipulation of protein degradation in the rumen or efficient use of N in the rumen is the most cost-effective strategy to control N losses to the environment (Tamminga, 1996). In this study, chitosan inclusion had no effect on urinary N excretion, whereas the higher the N intake associated with feeding, the higher the level of CP, which resulted in an increase in urinary excretion for those, offered the HP diets. Feeding the HP diets would have supplied a higher percentage of rumen-degradable protein (RDP) compared to those offered the LP diets, resulting in excess N for microbial protein synthesis (Colmenero and Broderick, 2006). In diets where low concentrations of CP are fed, the proportion of urea produced in the liver that is returned to the rumen via blood and saliva increases, resulting in lower proportions of urea excreted via the urine (Reynolds and Kristensen, 2008). Furthermore, the initial body weight of these animals indicated that these animals were mature in nature, had finished growing, and were in their finishing phase. Chitosan inclusion and the level of CP had no effect on retained N g d^{-1} (data not shown), highlighting that N intake does not interfere with muscle deposition in mature animals (Menezes et al., 2016).

Faecal N is primarily of microbial origin, with lesser amounts of undegraded feed protein and endogenous secretions (NRC, 2001). The increase in faecal N excretion associated with the animals that were offered chitosan is likely associated with the decrease in DM, OM, and CP digestibility. Previously, Mingoti et al. (2016) found that including chitosan in the diet of lactating dairy cows reduced faecal N excretion and they concluded that this was as a result of better utilization of N, which reached the small intestine as a result of changes in rumen fermentation caused by chitosan, correlating with improvements in protein digestibility. However, Goiri et al. (2010) reported a negative impact of chitosan on NDF digestibility in sheep and reduced VFA concentrations in faecal samples, suggesting that chitosan exercised the same antimicrobial action in the cecum as in the rumen, but to a greater extent.

5.5.3. Rumen fermentation parameters

The pH of the environment is vital to the antibacterial activity of chitosan. According to Kong et al. (2010), when pH is below the molecules pK_a (6.3-6.5), chitosan becomes polycationic, which causes electrostatic interaction between the chitosan and the anionic components of the microorganism's surface, while on the other hand, hydrophobic and chelating effects are responsible for antibacterial activity of chitosan when the environment is above the pK_a. Rumen pH is a critical factor in the normal and stable functioning of the rumen because of its profound effect on microbial populations and fermentation products, and on physiological functions of the rumen. The difference observed in ruminal pH was relatively minor and would likely not be biologically significant. Cellulolytic bacteria in the rumen are responsible for fibre digestion within the rumen and as ruminal pH starts to fall below 6.2 their activity starts to diminish (Mould et al., 1983). The negative correlation between ruminal pH and total VFA concentration highlights the pH-reducing potential of VFA

accumulation in the rumen (Dijkstra et al., 2012). There was no difference observed in the concentration of individual VFA or total VFA concentrations from the inclusion of chitosan in the diet. However, nutrient digestibility was decreased with the inclusion of chitosan, thereby reducing the VFA production potential in the diet. Ammonia has a high pKa value (9.21) and as a consequence, virtually all NH_3 is present in the rumen as NH_4^+. Production of NH_3 in the rumen can assist in the regulation of ruminal pH through the disposal of NH_4^+. Therefore, the extra supply of NH_3 from the degradation of amine groups in chitosan may explain the increase in ruminal pH associated with the inclusion of chitosan.

The higher ruminal NH_3 concentrations associated with feeding the HP diet were expected because of the increased dietary percentages of RDP (Colmenero and Broderick, 2006). As dietary CP increases, there is greater deamination of amino acids released from protein degradation, which elevates NH_3 (Sannes et al., 2002). The increase in iso-valeric acid associated with the LP diets are a consequence of the deamination and decarboxyl-ation of the branch-chained amino acids (Allison, 1970). The addition of chitosan to the HP diets increased NH_3 concentrations, as was similarly reported by Araújo et al. (2015), where increases in NH_3 concentrations were observed in steers fed chitosan. The increased concentrations of ruminal NH_3 in the HP+ diet suggest that this was likely due to an extra supply of NH_3 from the degradation of amine groups in chitosan and a lower uptake of NH_3 by the rumen microbes, rather than increased proteolysis (Belanche et al., 2016a). Kang-Meznarich and Broderick (1980) reported 1.94 to 5 mmol L^{-1} to be the optimum level of ruminal NH_3 concentration adequate for microbial synthesis and fibre digestion, suggesting the levels of ruminal NH_3 produced in the LP diets were below optimum, which might

explain why no differences were observed in ruminal NH_3 concentrations between the two LP diets.

Previous studies found that chitosan inclusion in ruminant diets increased ruminal propionic acid concentrations (Dias et al., 2017; de Paiva et al., 2017), while Araújo et al. (2015) observed decreases in ruminal acetic acid coupled with increases in propionic acid concentrations as a result of increased nutrient intake and digestibility. The shift in fermentation products within the rumen may be as a result of the degradation of chitosan within the rumen, with the remaining carbon skeleton used by certain bacteria (Chen et al., 2002). Though chitosan inclusion had no effect on ruminal VFA profiles in the current study, the negative effect on nutrient digestibility may have potentially affected VFA production as a result of inefficient eating and chewing efficiency (Haraki et al., 2018). In contrast to the previous studies mentioned, the A: P was substantially higher, reflecting the high NDF contribution from the GS offered, influencing both VFA concentrations and the feeding behaviour of the animals (Dado and Allen, 1995). However, the level of CP did affect the ruminal VFA profiles in this study. Feeding the higher level of CP resulted in lower ruminal acetic acid and higher propionic concentrations. Feeding the HP diets would have supplied a higher percentage of RDP (Colmenero and Broderick, 2006), which has been shown to increase propionic acid and decrease acetic acid concentrations in the rumen (Baumann et al., 2004).

5.6 Conclusions

Increasing protein supplementation excess to requirements can result in elevated urinary N excretion, which has negative environmental consequences. The inclusion of chitosan showed no potential in reducing N excretion, while having a negative

effect on nutrient digestibility. As chitosan is not a single compound, but rather a series of different compounds that differ in degree of acetylation and other physiochemical characteristics, further studies are necessary to determine if chitosan has a role in modifying rumen fermentation.

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Chapter 6

Effect of dietary crude protein level and brown seaweed source on ruminal fermentation parameters *in vitro* using the rumen simulation technique (RUSITEC)

6.1 Abstract

Mitigation strategies to reduce ammonia (NH_3) emissions from the ruminant sector are required. Reducing the amount of crude protein (CP) in ruminant diets has shown to reduce NH_3 emissions by reducing urinary N excretion. Feeding terrestrial tannins also reduces urinary N excretion, therefore, feeding brown seaweeds, which are a source of phlorotannins (PT), may offer potential also. However, knowledge surrounding the effect of brown seaweeds on rumen function and N metabolism is limited. The objective of this study was to evaluate the effects of CP level and brown seaweed inclusion, on rumen fermentation *in vitro* using the RUSITEC system, with a 2 × 4 factorial study. The factors were: CP level (12.08% LP vs 16.70% HP) and brown seaweed species (no seaweed CO, *Alaria esculenta* ALE *Ascophyllum nodosum* ASC, *Himanthalia elongate* HIM, with seaweed species supplemented at 50g kg dry matter^{-1} (DM). With the exception of N digestion, CP level or brown seaweed species had no effect on nutrient digestion ($P > 0.05$). Feeding HP supported higher N digestion, while ALE and HIM reduced N digestion ($P < 0.05$). Brown seaweed species had no effect on total gas production or methane (CH_4) production ($P > 0.05$), whereas HP increased CH_4 production ($P < 0.05$). Brown seaweeds species reduced NH_3 concentrations in the overflow vessels ($P < 0.05$) while CP level had no effect ($P > 0.05$). There was a CP x brown seaweed species interaction ($P < 0.05$) for NH_3 concentrations in the overflow vessels with lower NH_3 concentrations in overflow vessels ($P < 0.05$) from HP ALE compared to HP CO, whereas vessels that received LP HIM had lower NH_3 concentrations compared to vessels that received LP CO ($P < 0.05$). Vessels that received HP ALE had higher concentrations of acetic acid, and total volatile fatty acids compared to vessels offered HP ASC, HP HIM, and HP CO ($P < 0.05$). Under

in vitro conditions, regardless of the CP level in diets offered, all brown seaweed species reduced NH_3 concentration, while offering diets lower in CP resulted in lower CH_4 production.

6.2 Introduction

Ruminant production systems are facing challenging emissions reductions targets (DAFM, 2021) due to their negative impact on the environment, as they are a major contributor to atmospheric ammonia (NH_3) emissions, a potentially harmful transboundary gas (Ndegwa et al., 2008). Agriculture accounts for 99.1% of total NH_3 emissions in Ireland, with animal manures accounting for 90% of these total emissions, largely driven by the cattle sector (EPA, 2021). Nitrogen (N) use efficiency is relatively low in ruminants, with 65-90% of ingested N excreted via the urine and faeces. Urea is the main form of N excreted in the urine which can be hydrolysed and volatised as NH_3 (Hristov et al., 2011), leading to NH_3 gas emissions. When ruminants consume high quality forages, most of the proteins therein are soluble and rapidly degraded in the rumen, leading to inefficient use of N for microbial synthesis (Min et al., 2005), and increased NH_3 levels within the rumen which can lead to increases in urinary N excretion (Patra and Saxena, 2011). Feeding dietary crude protein (CP) in excess of the animal's requirements, leads to increases in the amount of N excreted (Colmenero and Broderick, 2006). As the level of CP increases in the diet, urinary N excretion increases at a greater rate than faecal N and strategies that reduce CP in the diet are generally associated with a reduction in urinary N output. Animal performance can be maintained with reduced dietary CP levels;

Leonardi et al. (2003) and Colmenero and Broderick (2006) observed no affect in milk production when dietary CP content was reduced from 18.9% to 16.1% and 19.4% to 13.5% respectively. Similarly, reducing the CP content in cattle finishing diets from 14% to 12% and 15% to 11% had no negative effect on animal performance (Cole et al., 2003; Ludden et al., 2002).

Various nutritional strategies have been evaluated in the quest for alternative natural products that can modify ruminal fermentation to mitigate N excretion and enteric methane (CH_4) emissions from ruminants (Grossi et al., 2019). A substantial amount of research has focused on the role that secondary plant compounds (essential oils, saponins, and tannins) play in modifying ruminal fermentation (Wallace et al., 2002; Patra and Saxena, 2011; Cobellis et al., 2016). It has long been recognised that terrestrial tannins (condensed and hydrolysable) have the ability to reduce the extent of protein degradation in the rumen by direct inhibition of microbial activity and indirectly by forming tannin-protein complexes in the rumen (Patra and Saxena, 2011). They inhibit the growth and activities of proteolytic bacteria and have been shown to shift the site of N metabolism from the rumen to the lower digestive tract and large intestine (Min et al., 2003; de Klein and Eckard, 2008), shifting N excretion from urinary to faecal and increasing overall N utilization (Grainger et al., 2009).

Seaweeds such as brown algae (Phaeophyceae), red algae (Rhodophyceae) and green algae (Chlorophyceae) are naturally rich in minerals, trace elements and amino acids and consequently have a long history of use as a livestock feed (Balasse et al., 2005; Tayyab et al., 2016). The nutritional value varies considerably, depending on species, time of

harvesting, habitat, and external conditions such as water temperature, light intensity, and the nutrient content of the water (Molina-Alcaide et al., 2017). Brown algae have a lower nutritional value than red and green algae due to their lower protein content and higher mineral content, but contain several bioactive compounds (Makkar et al., 2016). As an important resource of bioactive compounds, they can produce a huge diversity of secondary metabolites characterized by a broad spectrum of biological activities such as antimicrobial, antibacterial, antioxidant, antiviral and antifungal (Val et al., 2001; Yuan et al., 2006; Wang et al., 2008). Compared to the red and green species, brown seaweeds are the only species able to produce high levels of Phlorotannins (PT) as a result of phloroglucinol polymerisation (Wang et al., 2008). Phlorotannins in *Ascophyllum nodosum* (ASC) have been found to possess strong activity against ruminal microbes and it is thought that this antimicrobial activity might be species-dependent (Wang et al., 2006, 2008). It has also been reported that ASC promoted a decrease in N degradability (-24%) (Belanche et al., 2016b), with similar results found with PT from *Laminaria digitata* (Vissers et al., 2018). The effect of terrestrial tannins has been studied extensively regarding their benefits on ruminant nutrition (Min and Hart, 2003); however, knowledge on the effect of PT on rumen function is still in its infancy.

It is hypothesized that inclusion of brown seaweed species will inhibit ruminal protein degradation and reduce ruminal NH_3 concentrations without affecting rumen fermentation in diets differing in CP content *in vitro.* Therefore, the objective of this study was to evaluate the effects of dietary CP level and brown seaweed species, and their interactions

rumen fermentation *in vitro* using the artificial Rumen Simulation Technique (RUSITEC).

6.3 Materials and methods

6.3.1 Animals and experimental licencing

All animal procedures described in this experiment were approved by the Animal Research Ethics Committee (AREC) (approval number: AREC-E-17-14-Pierce) in University College Dublin (UCD) and conducted under the European Directive 2010/63 EU and S.I. No. 543 of 2012. Any person who carried out procedures on experimental animals, during this experiment, were authorised to do so by means of individual authorisation from the Health Products Regulatory Authority (HPRA). This experiment was conducted at UCD Lyons Research Farm, Celbridge, Naas, Co. Kildare, Ireland. W23 ENY2 (53^0 17' 56" N, 6^0 32' 18" W).

6.3.2 Experimental procedure, rumen inoculum, diets

Rumen inoculum was sourced from four rumen-cannulated lactating dairy cows (703 ± 29 kg BW) fed a diet consisting of predominately perennial ryegrass pasture plus 3 kg concentrate on a fresh weight basis.

The *in vitro* experiment consisted of a single incubation period using three RUSITEC systems with eight vessel per system (Sanshin Industrial Co. Ltd, Yokohana, Japan), which were used to simulate the rumen environment. Each vessel had an effective volume of 800 ml and were kept at 39 °C under permanent vertical agitation as described by Czerkawski and Breckenridge (1977).

Each vessel was considered an experimental unit, in a 2 × 4 factorial arrangement. The factors were CP level (12.08% LP vs 16.70% HP) and brown seaweed species (no seaweed CO, *Alaria esculenta* ALE, *Ascophyllum nodosum* ASC, *Himanthalia elongate* HIM), with brown seaweed species supplemented at 50 g kg dry matter^{-1} (DM). *Ascophyllum nodosum* was harvested during spring 2019 from the middle intertidal zone (54.973643, -8.488973), while both *Alaria esculenta* and *Himanthalia elongate* were harvested during summer 2019 from the low intertidal zone (54.973643, -8.488973 and 54.970406, -8.460702 respectively).

Table 6.1 Ingredient composition and chemical composition of dietary treatment.

Crude protein level[1]	HP				LP			
Seaweed[2]	CO	ALE	ASC	HIM	CO	ALE	ASC	HIM
Ingredient composition, % DM								
Maize meal	48.75	48.75	48.75	48.75	50	50	50	50
Maize distillers'	1.25	1.25	1.25	1.25	-	-	-	-
Grass silage	50	50	50	50	50	50	50	50
Chemical composition, % DM								
Dry matter	57.69	57.69	57.69	57.69	57.58	57.58	57.58	57.58
Crude protein	16.70	16.70	16.70	16.70	12.08	12.08	12.08	12.08
Neutral detergent fibre	24.94	24.94	24.94	24.94	24.70	24.70	24.70	24.70
Acid detergent fibre	13.29	13.29	13.29	13.29	13.21	13.21	13.21	13.21
Starch	31.68	31.68	31.68	31.68	32.5	32.5	32.5	32.5

[1]HP, High CP (16.70%); LP, low CP (12.08%).
[2]Brown seaweed species: ALE *Alaria esculenta*, ASC *Ascophyllum nodosum*, HIM *Himanthalia elongate* at inclusion 50 g kg DM^{-1}.

Once harvested, all brown seaweed species were air dried at 30 °C and milled to pass through 1 mm screen. The *in vitro* basal diet consisted of 50: 50 forage: concentrate on a DM basis (Table 6.1). Concentrates in the LP diet consisted of maize meal while the concentrate in the HP diets consisted of 75% maize meal and 25% maize distillers' grains.

The *in vitro* incubation period lasted for 16 d, with the first 10 d for microbial adaptation and fermentation stabilisation and the last 6 d for sampling. Collection of rumen inoculum (fluid and digesta) took place after morning milking at 0900 on d 0. Solid digesta and rumen inoculum were collected, with the inoculum strained through four layers of cheesecloth. Rumen inoculum from all cows was pooled, flushed with carbon dioxide (CO_2), and incubated at 39 °C before being transferred to the RUSITEC vessels within 45 minutes of collection. Each vessel was inoculated with 450 mL of rumen inoculum and 350 mL of anaerobic artificial saliva (Mc Dougall, 1948). Dietary treatments were added to each vessel in nylon bags (100-µm pore size; 5 × 10 cm concentrate; 10 × 20 cm forage; ANKOM ^{TM} Technology, Macedon, NY, USA); 70 g of rumen solid digesta and incubated in each vessel for 1 d to provide solid associated bacteria, a second bag containing 10 g DM grass silage (GS) and a third concentrate bag containing 10 g DM concentrate plus brown seaweed species treatment. The concentrate feed component was ground through a 1 mm sieve and the forage component was chopped to 2-4 cm in length using a bowl chopper. After 24 h, each vessel was opened and two of the initial three bags removed – bag containing rumen digesta solids and the bag containing the concentrate plus brown seaweed species of interest – squeezed and washed in 50 ml of artificial saliva. The liquid fractions of the washings were returned to the vessels and two new nylon bags, containing

10 g DM GS and 10 g DM of concentrate plus brown seaweed species treatment of interest were inserted into the fermentation vessels. On subsequent days, the nylon bag containing grass silage that had been in the vessel for 48 h was replaced with a new nylon bag containing GS, and the nylon bag containing the concentrate of interest, which had been in the vessel for 24 h was replaced as described above.

Artificial saliva was prepared daily and was continuously infused at a rate of 640 ml d^{-1} (dilution rate of 3.33% h^{-1}) to prevent the wash out of rumen microbes, using a multichannel peristaltic pump (Watson-Marlow 500 series, Cornwall, UK). The displaced effluent and fermentation gasses from each fermentation vessels were collected into effluent bottles and gas collection bags, respectively.

6.3.3 Sampling

Dry matter degradation, gas production, CH_4 and outflow of fermentation products were measured on d 11, 12, 13, 14, 15 and 16. Overflow vessels were kept in a water bath maintained at 2 °C to stabilise fermentation products. Samples of outflow liquor (4 ml) were collected using an automatic pipette and mixed with 1 ml TCA (500 g L^{-1} trichloroacetic acid) and stored at -20 °C for subsequent VFA and NH_3 analysis. Fermentation gases were collected in reusable polyethylene gas-tight bags fitted with one-way valves that were attached to each outflow vessel. Total gas production was measured using a DC-1 dry gas test meter (Sinagawa Corp.; Tokyo, Japan), and CH_4 percentage was determined using a GC100 portable CH_4 reader (ADC Gas Analysis; Hoddeston, UK). Nylon bags were collected and rinsed with ice-cold water. Feed residues in the nylon bags were washed in a domestic washing machine using the cold rinse cycle in the absence

of detergent (30 min) to remove loosely attached bacteria in the bags. The feed residue was then dried in a 55 °C forced air oven for 48 h and weighed. Feed DM digestibility was calculated as the amount of material that disappeared from the nylon bags after 24 h and 48 h of incubation, for concentrates and GS, respectively. Chemical composition of the dried incubation residues was used to calculate digestibility of feed components as follows:

(sample end weight – empty bag weight) / (sample start weight – empty bag weight)

6.3.4 Chemical analysis

Dried samples of GS, concentrates and feed residues were dried at 55 °C for 48 h in a forced air oven, ground in a hammer mill fitted with a 2 mm screen (Lab Mill, Christy Turner, Suffolk, UK) and stored for DM determination. The DM content of samples was determined after drying overnight at 105 °C (minimum 16 h) (method 930.15; AOAC International, 2005). Ash concentrations were determined by complete combustion in a muffle furnace (Nabertherm Gmbh, Lilienthal, DE) at 550 °C for 5 h (AOAC International, 2005a). Starch was determined using the Megazyme Total Starch Assay Procedure (Product no: K-TSTA; Megazyme International Ireland LTD, Wicklow, IE; McCleary et al., 1994). The N content was determined using a LECO FP 528 instrument (Leco Corp, St. Joseph, Michigan, US) (AOAC International, 2005b).

Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the method of Van Soest et al. (1991) adopted for the use in the ANKOM ^{TM} 220 Fibre Analyser (ANKOM ^{TM} Technology, NY, US). Concentrate samples were analysed with a thermos-stable α-amylase and 20 g of Sodium sulphite ($NaSO_3$) was added to neutral

detergent solution (NDS), while GS and incubation residues were analysed with NDS only. Neutral detergent fibre and ADF are expressed inclusive of residual ash. Gross energy (GE) of feed were determined by bomb calorimetry (Parr 1281 Bomb Calorimeter, Parr Instrument Company, Illinois, US). Ether extract (EE) was determined using Soxtex instruments (Tecator, Hoganas, SE) and light petroleum ether.

Overflow liquor samples were thawed for 16 h at 4 °C and centrifuged at 1,800 × *g* for 10 min at 4 °C. One ml of supernatant was drawn off, diluted 1 in 5 with dH2O, and centrifuged at 1,800 × *g* for 15 min at 4 °C. From this, 200 µl supernatant was drawn off and NH_3 concentrations were determined using the phenolyhpochlorite method of Weatherburn (1967). For VFA analysis, a sample containing 250 µl of supernatant was drawn off into a separate test tube and diluted with 3.75 ml of dH_2O and 1 ml of internal standard (0.5 g 3-metyl-n-valeric acid in one litre of 0.15 M oxalic acid). Following centrifuging for five minutes, 260 × *g* for 21 °C, a subsample was filtered through a 0.45 micrometre (µm) filter (Cronus Syringe filter PTFE 13 mm; SMI-LabHut Ltd., Maisemore, Gloucester, UK) into a four ml GC vial (Thermo Scientific, Langerwehe, Germany) and frozen at -20 °C until VFA analysis. One µl of sample was injected via an auto sampler on a Varian gas chromatograph (GC) 3800 with a 25 m × 0.53 mm i.d. megabore column (coating CP-Wax 58 (FFAP) – CB (no. CP7614), (Varian, Middelburg, Netherlands). The initial injector temperature was 75 °C, rising immediately to 95 °C, temperature then increased at a rate of 3 °C min^{-1} until the temperature reached 200 °C, and was then held for 50 s. Nitrogen was used as a carrier gas. Column pressure was kept at 2.3 psi and the column rate was 8.1 ml min^{-1}. Estimates of hydrogen (H) recovery within

each vessel were calculated based on the concentration of individual VFAs with the exclusion of hydrogen gas (H_2) (Ungerfeld, 2015).

Total phenolic content in the brown seaweed species was determined according to the method of Stankovic (2011) (Table 6.2). The total phenolic contents in the examined extracts using the Folin-Ciocalteu's reagent is expressed in terms of phloroglucinol equivalents (the standard curve equation: $y = 4775.6x-0.1114$; $R^2 = 0.9948$). The values obtained for the concentration of total phenols are expressed as mg of phloroglucinol g^{-1} extract. Each value is the average of three measurements of absorbance at 765 nm +/- the standard deviation.

Table 6.2 Chemical composition (g kg DM^{-1}) and concentration of phlorotannins in brown seaweed species.

Seaweed[1]	ALE	ASC	HIM
Dry Matter	829.8	857.9	829.6
Ash	214.3	182.9	278.4
Nitrogen	17.22	4.64	5.91
Crude protein[2]	71.81	23.80	24.64
Carbon	313.0	373.5	367.9
NDF[3]	141.4	198.5	132.0
Total phenols[4]	28.50	59.85	30.44
Phlorotannins	23.44	52.16	23.77

[1]Brown seaweed species: ALE *Alaria esculenta*, ASC *Ascophyllum nodosum*, HIM *Himanthalia elongate.*
[2]Protein correction factor: nitrogen × 5.39.
[3]Neutral detergent fibre.
[4]Total phenolic content in the extracts expressed in terms of phloroglucinol equivalents (mg of phloroglucinol g^{-1} extract).

6.3.5 Statistical analysis

Data were analysed using the PROC MIXED procedure of Statistical Analysis Software (SAS v9.4, Inst. Inc., Cary NC, USA). Normal distribution and homogeneity of variance were analysed using the UNIVARIATE procedure. Data that were not normally distributed were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box-Cox transformation analysis using the TRANSREG procedure of SAS. Fixed effects in the model included brown seaweed species and CP level and their interaction. Vessel was included as a random effect. Where interactions were not significant, this term was excluded from the model. Statistically significant differences between least squares means were tested using the PDIFF command incorporating the Tukey test for pairwise comparison of treatment means. *In vitro* data collected at different times after feeding were analysed using the PROC MIXED procedure for repeated measures. The model contained the same fixed effects as before, except that day or time after feeding and its interaction with the main effects were included. Statistical significance was assumed at a value of $P < 0.05$ and a tendency toward significance assumed at a value of $P > 0.05$ but $P < 0.10$.

6.4 Results

Crude protein level and brown seaweeds species had no effect ($P > 0.05$) on feed degradation (Table 6.3). Offering HP resulted in a higher ($P < 0.05$) percentage of N degraded whereas offering ALE and HIM reduced ($P < 0.05$) N degradation.

There was a CP × brown seaweed species interaction for pH in the overflow vessels ($P < 0.001$). Vessels containing HP ALE had the lowest overflow pH compared to the vessels that received HP ASC, HP CO, and HP HIM, and vessels that received LP ALE, LP CO, and LP HIM. High CP diets resulted in the highest pH in the overflow vessels ($P < 0.05$), while brown seaweed species decreased overflow pH ($P < 0.01$).

There was a CP x brown seaweed species interaction ($P < 0.05$) for NH_3 concentration (Table 6.4). Vessels that received HP ALE had lower NH_3 concentrations in overflow vessels ($P < 0.05$) compared to HP CO, whereas vessels that received LP HIM had lower NH_3 concentrations compared to vessels that received LP CO ($P < 0.05$). Crude protein had no effect on overflow NH_3 concentration ($P > 0.05$). All three brown seaweed species offered reduced NH_3 concentrations in overflow vessels ($P < 0.05$).

Table 6.3 Effect of supplementing different crude protein diets with different brown seaweeds on nutrient digestibility *in vitro* using the rumen simulation technique (RUSITEC).

CP level[1]	HP				LP					*P*-value[1]		
Seaweed[2]	CO	ALE	ASC	HIM	CO	ALE	ASC	HIM	SEM	CP	SW	CP×SW
Disappearance												
Dry matter	74.60	77.78	73.92	71.72	77.62	73.17	77.14	76.83	2.321	0.311	0.881	0.203
Organic matter	73.94	77.16	73.33	70.79	77.34	72.63	76.88	76.29	2.438	0.268	0.847	0.215
Nitrogen	86.82	85.70	85.98	82.89	86.05	79.91	84.86	83.07	1.206	0.043	0.018	0.102
NDF[3]	56.14	61.43	55.37	51.85	60.27	48.96	59.03	65.25	6.701	0.653	0.957	0.294
Starch	76.01	81.34	77.90	74.37	76.47	78.06	78.31	76.21	1.460	0.892	0.044	0.412

[1]HP, High CP (16.5%); LP, low CP (12.08%).
[2] Brown seaweed species : ALE *Alaria esculenta*, ASC *Ascophyllum nodosum*, HIM *Himanthalia elongate* at inclusion 50 g kg DM^{-1}.
[3]Neutral detergent fibre.

There was a CP × brown seaweed species interaction for acetic acid, propionic acid, butyric acid, and total VFA concentrations ($P < 0.01$). Vessels that received HP ALE had higher acetic acid concentrations compared to vessels that received HP ASC, HP HIM, HP CO, while vessels that received LP ALE had lower acetic acid concentrations compared to vessels that received LP ASC ($P < 0.05$). Vessels that received HP ALE had higher concentrations of propionic acid compared to vessels that received HP CO ($P < 0.01$), and LP ALE ($P < 0.05$). Vessels that received HP ALE had higher concentrations of butyric acid compared to vessels that received HP CO ($P < 0.05$). Vessels that received HP ALE had a higher total VFA concentrations compared to vessels that received HP ASC, HP CO, HP HIM, while vessels that received LP ALE had lower concentrations compared vessels that received LP ASC ($P < 0.05$). Crude protein and brown seaweed species had no effect on acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, and total VFA concentrations and on the acetic acid: propionic acid in the overflow vessels ($P > 0.05$). The level of CP offered had no effect on valeric acid concentrations ($P > 0.05$), whereas brown seaweed species inclusion tended to increase valeric acid concentrations ($P < 0.10$). Vessels that received HP had the highest concentrations of iso-valeric acid ($P < 0.05$), while brown seaweed species inclusion had no effect ($P > 0.05$).

The inclusion of brown seaweed species had no effect on total gas produced, volume of CH_4, CH_4 percentage, and the concentration of CH_4 ($P > 0.05$) (Table 6.4). There was a CP × brown seaweed species interaction for volume of gas produced ($P < 0.001$). Vessels containing HP ALE produced higher volumes of gas compared to CO HP ($P < 0.001$).

Vessels that received LP produced the lowest percentage of CH_4 recovered from gas bags daily ($P < 0.05$), while vessels that received HP produced the highest volume of CH_4 ($P < 0.05$). The concentration of CH_4 produced was lowest for vessels that received the LP diets ($P < 0.05$). However, when CH_4 was expressed per g DOM, neither the level of CP offered, or the inclusion of brown seaweed species had an effect ($P > 0.05$).

The percentage of metabolic hydrogen (H) recovered was not affected by CP level or the inclusion of brown seaweed species ($P > 0.05$). There was a CP × brown seaweed species interaction for metabolic H released and accepted ($P < 0.001$). More metabolic H was released and accepted in the vessels containing HP ALE compared to vessels containing HP ASC, HP CO, HP HIM, and LP ALE. Furthermore, vessels that were offered HP CO had lower levels of metabolic H accepted compared to the vessels offered LP ASC, LP CO, and LP HIM.

Table 6.4 Effect of supplementing different crude protein diets with different brown seaweeds on ruminal fermentation parameters *in vitro* using the rumen simulation technique (RUSITEC).

CP level[1]	HP				LP					*P*-value		
Seaweed[2]	CO	ALE	ASC	HIM	CO	ALE	ASC	HIM	SEM	CP	SW	CP×SW
pH	6.65	6.28	6.60	6.57	6.46	6.49	6.39	6.48	0.043	0.0376	0.0001	0.001
Concentration (mmol L $^{-1}$)												
NH_3	3.22	2.33	2.80	2.93	3.37	2.99	2.57	2.49	0.213	0.890	0.01	0.055
Acetic	76.10	96.93	78.99	79.77	84.67	74.65	93.61	90.23	4.087	0.364	0.478	<0.001
Propionic	20.40	25.11	21.70	22.05	23.26	20.92	23.62	23.99	0.916	0.494	0.422	<0.001
Butyric	13.35	16.11	13.81	13.80	14.35	13.75	15.29	14.41	0.574	0.747	0.231	0.005
Iso butyric	0.84	0.88	0.75	0.76	0.87	0.70	0.81	0.86	0.042	0.891	0.315	0.710
Valeric	3.96	4.45	4.67	4.28	3.91	4.68	4.28	4.01	0.265	0.531	0.070	0.663
Iso valeric	2.93	3.00	3.09	3.05	3.10	2.41	2.83	2.76	0.152	0.027	0.199	0.116
Total VFA[3]	117.8	146.4	123.0	123.7	129.3	117.1	139.4	136.4	5.28	0.452	0.396	0.001
A: P[4]	3.71	3.88	3.66	3.60	3.68	3.58	4.00	3.77	0.106	0.529	0.513	0.210
Total gas (L d^{-1})	1.51	2.26	2.02	1.85	2.10	1.71	1.89	1.90	0.141	0.895	0.565	0.001
Methane (L d^{-1})	0.29	0.34	0.37	0.30	0.31	0.27	0.28	0.26	0.026	0.012	0.355	0.167
Methane (%)	5.30	6.74	5.46	6.38	7.32	6.57	7.08	7.55	0.492	0.001	0.463	0.134
Methane (mmol d^{-1})	11.78	14.11	14.40	11.75	11.62	11.15	11.56	11.78	1.018	0.041	0.506	0.278
Methane mmol g DOM^{-1})	0.93	1.00	1.04	0.98	0.81	0.79	0.75	0.76	0.142	0.051	0.994	0.944

[1]HP, High CP (16.70%); LP, low CP (12.08%).

[2]Brown seaweed species : ALE *Alaria esculenta*, ASC *Ascophyllum nodosum*, HIM *Himanthalia elongate* at inclusion 50 g kg DM^{-1}.

[3]Volatile fatty acids.

[4]Acetic acid: Propionic acid.

[5]Metabolic hydrogen stoichiometric calculated based on VFA production (Ungerfeld, 2015).

Table 6.4 (cont'd) Effect of supplementing different crude protein diets with different brown seaweeds on ruminal fermentation parameters *in vitro* using the rumen simulation technique (RUSITEC).

CP level[1]	HP				LP					*P*-value		
Seaweed[2]	CO	ALE	ASC	HIM	CO	ALE	ASC	HIM	SEM	CP	SW	CP×SW
[H] *produced*[5] (mmol d^{-1})	238.2	296.8	248.9	249.6	263.9	239.3	285.5	274.3	10.76	0.334	0.386	<0.001
[H] *incorporated*[5] (mmol d^{-1})	82.26	109.6	92.16	89.62	100.9	89.92	103.8	104.1	3.59	0.015	0.137	<0.001
[H] *recovery*[5] *(%)*	34.95	36.45	37.85	36.66	38.76	37.58	36.02	37.55	1.121	0.209	0.997	0.125

[1]HP, High CP (16.70%); LP, low CP (12.08%).

[2]Brown seaweed species : ALE *Alaria esculenta*, ASC *Ascophyllum nodosum*, HIM *Himanthalia elongate* at inclusion 50 g kg DM^{-1}.

[3]Volatile fatty acids.

[4]Acetic acid: Propionic acid.

[5]Metabolic hydrogen stoichiometric calculated based on VFA production (Ungerfeld, 2015).

6.5 Discussion

Phlorotannins are a polyphenolic compound found exclusively in brown seaweed species and their content is influenced by numerous factors, such as habitat, location and harvest season (Makkar et al., 2016). Seaweeds that are found on the middle intertidal zone have a higher PT content than those found on the low intertidal zone, which is supported by the PT content found in the brown seaweed species used in this study. The level of PT found in ASC was 55% higher compared to the levels found in ALE and HIM.

The inclusion of brown seaweed species and CP level had no effect on nutrient digestibility (DM, OM, and NDF) and was reflected in values obtained for total gas production and VFA results in this study. It is well documented that a shortage of fermentable N in the rumen can have negative effects on nutrient digestibility (DM, OM, and NDF), low N inhibits microbial growth and thus, there is not enough bacteria for fibre digestion, (Doreau et al., 1990). However, N was not a limiting factor in this study as observed by the ruminal NH_3 concentrations (Kang-Meznarich and Broderick, 1980). Wang et al. (2008) observed that the properties of PT in *Ascophyllum nodosum* were similar to hydrolysable tannins such as tannic acid, having a negligible impact on ruminal bacteria involved in starch digestion. While the PT and total phenol content was similar between *Alaria esculenta* and *Himanthalia elongate* in this study, starch digestion was promoted in vessels that received *Alaria esculenta*. The exact mode of action of PT on the inhibition of carbohydrate digestibility has not been fully elucidated but may be similar to terrestrial tannins, where its binding ability is independent of pH, and may be influenced by the tannins water solubility (Seigler, 1998) and molecular weight (Aboagye and Beauchemin, 2019). The large variation in molecular weight associated with PT, 126 to 650,000 Da (Aboagye and Beauchemin,

2019) may explain the variations observed between brown seaweed species with similar PT content.

Ruminal pH is one of the most important factors in the normal and stable functioning of the rumen because of its profound effect on microbial populations and fermentation products, and on physiological functions of the rumen. Fibrolytic bacteria are very sensitive to changes in ruminal pH and their activity diminishes especially below pH 6.0 (Calsamiglia et al., 2012). Nevertheless, the pH herein was in the range of 6.28 to 6.65, which is optimum for fibre digestion (Mouriño et al., 2001). Considering the forage: concentrate and starch levels fed in the experimental diets, the stability of pH values observed within treatments can be explained by the continuous infusion of buffered artificial saliva (Maia et al., 2019). Ammonia is a very potent buffer (high pK_a value; 9.21), which might explain the increase in ruminal pH associated with the vessels that received HP. However, there was no difference observed in NH_3 concentrations between vessels offered the different levels of CP. Compared to other species, brown seaweeds have an abundant supply of minerals (Makkar et al., 2016), particularly potassium and sodium, which are exhibited in the high ash content (Jard et al., 2013). However, the buffering capacity of each brown seaweed species linked to the ash fails to substantiate the pH values observed. Vessels that received HP ALE yielded the lowest pH values despite the higher ash value compared to ASC (214.3 g kg^{-1} versus 182.9 g kg^{-1} respectively). Ruminal pH will drop as the concentration of VFA, or lactic acid increase as a result of microbial fermentation of carbohydrates and protein following a meal, with ruminal VFA negatively related to ruminal pH (Dijkstra et al., 2012), explaining the low pH values in vessels that received HP ALE mirrored by the high acetic acid, propionic acid, butyric acid, and total VFA concentrations.

Ammonia concentrations observed in this study were not affected by the level of CP offered, despite the difference in CP concentration between HP and LP diets, with all values adequate for microbial synthesis and fibre digestion (Kang-Meznarich and Broderick, 1981). Both experimental diets were supplemented with the same energy source, maize meal, and contained similar levels of starch (HP 304.6 & LP 312.0 g kg^{-1}). Carbohydrates fermented in the rumen provide most of the energy (ATP) that drives microbial protein synthesis. Non-structural carbohydrates in ruminant diets such as starch ensure an adequate energy supply in the rumen, improving microbial protein synthesis and N utilisation (Mabjeesh et al., 1997). However, the inclusion of brown seaweed species herein did reduce NH_3 concentrations but not sufficiently to affect nutrient digestibility, as the binding strength of the tannin-protein interactions determines the responses of tannins on protein digestibility (Mueller-Harvey, 2006). The inclusion of brown seaweed species reduced NH_3 concentrations (-18%) compared to the control. In a dose response study, Wang et al. (2008) observed similar reductions in NH_3 concentrations (-15%) with PT obtained from *Asophyllum nodosum*, while Belanche et al. (2016) observed no reductions in NH_3 concentrations with the brown seaweed species *Asophyllum nodosum* and *Laminaria digitata* included at 50g kg DM^{-1}. Among all seaweeds species, brown seaweeds are the only ones able to produce high levels of PT, suggesting that the degradability of feed protein was inhibited as a result of the formation of PT-protein complexes, similar to protein complexes formed with terrestrial tannins (Min et al., 2004). Ammonia concentrations were reduced when PT obtained from *Asophyllum nodosum* (Wang et al., 2009) and *Laminaria digitata* (Vissers et al., 2018) were included in a batch culture system, but negated when incubated together with polyethylene glycol. Moreover, reduction in NH_3 concentrations may have resulted from the inhibition of the digestive activity of

rumen microbes by PT in addition to the direct protective effect of PT on dietary protein (Wang et al., 2008).

Microbial fermentation of structural and non-structural carbohydrates in the rumen is of vital importance as it supplies 70% of the ruminant's energy supply (Bergman, 1990). The inclusion of brown seaweed species and the level of CP offered had no effect on acetic acid, propionic acid, butyric acid and total VFA concentrations, which is in line with the results observed for nutrient digestibility (DM, OM, and NDF) and total gas production. The levels of PT obtained from the brown seaweed species used in this study were sufficient to form protein-phenol complexes (Min and Hart, 2003) but inadequate to bond with fibre and carbohydrates and reduce CH_4 and total gas production. According to Patra and Saxana (2011), in diets with high concentrations of terrestrial tannins, tannins that remain free after binding to proteins may reduce fibre digestion by complexing with lignocellulose (Barry and Manley, 1986), thus preventing microbial digestion or by directly inhibiting cellulolytic microorganisms (Patra and Saxena, 2009) and activities of fibrolytic enzymes (Bae et al., 1993) or both. The carbohydrates contained in seaweeds contain complex polysaccharides found in the cell wall and vary amongst species (Makkar et al., 2016) with laminarin, mannitol, alginic acid, fucoidans and cellulose the main polysaccharides found in brown seaweed species (Sterner and Edlund, 2016).

The higher acetic acid, propionic acid, butyric acid and total VFA concentrations observed in vessels that received HP ALE may be attributed by the low contents of starch (Percival, 1979), cellulose in macroalgae and the high content of complex cell wall polysaccharides (Makkar et al., 2016), that differ among macroalgae classes, and even among species within classes (Ruocco et al., 2016). The interactions that were observed in VFA concentrations in overflow vessels between CP level and brown

seaweed species are likely due to possible differences in PT content among brown seaweed species, suggesting that formation of PT-enzyme complexes maybe the main factor which limits cellulase activity (Wang et al., 2008). While ALE contained the highest CP content, which was 66% higher than ASC and HIM, its effect on the overall CP content was minimal and may not be biologically important. Furthermore, ASC contained the highest phenol and PT content to form PT-protein complexes with dietary protein, which would not be attacked by bacteria (Wang et al., 2008).

The increased concentrations of iso-valeric acid observed in vessels that received HP was not unexpected as maize distillers was added to increase the CP content of the HP diets, while simultaneously increasing rumen degradable protein (RDP) (NRC, 2001). Increasing the CP and RDP content of the diet would lead to greater deamination of amino acids from protein degradation. Degradation of certain amino acids in the rumen results in the production of branched chain VFA (iso-acids) and can be used as an index of protein degradation (Hoover et al., 1986; Molina-Alcaide et al., 2017). Proportions are difficult to interpret because they are acquired and utilised by cellulolytic bacteria for growth, with observed concentrations the balance between N degradation and N used by the bacteria for microbial protein synthesis (Hume, 1970; Dijkstra et al., 1993). The higher concentrations of iso-valeric acid associated with vessels that received HP diets may indicate that the energy available for the maintenance of bacteria in the HP diets was limiting (Wallace et al., 1997). As feed is fermented in the rumen by the rumen micro-organisms, metabolic hydrogen [2H] is released, and the fermentation pathway to which this [2H] is then directed will determine how energetically beneficial it is to the animal (Guyader et al., 2017). As brown seaweed species inclusion had no inhibitory effect on methanogenesis, the higher [2H] released and incorporated associated with vessels that received HP ALE

suggests that the higher propionic acid concentrations observed was a consequence of enhanced starch digestion rather than a re-direction of [2H] to propionic acid production (Ungerfeld, 2015).

The findings to date in the literature on the anti-methanogenic effects of brown seaweed species inclusion in ruminant diets is equivocal. The lack of effect observed from brown seaweed species inclusion on total gas production and CH_4 emissions are in accordance with the results observed for nutrient digestibility, as gas and CH_4 production derive from DM and OM digestion. In a similar study, Belanche et al. (2016) observed no effect in *in vitro* CH_4 emissions with *Asophyllum nodosum* and *Laminaria digitata* included at 50 g kg DM^{-1}, while Maia et al. (2019) observed no effect on CH_4 emissions with seaweeds at higher inclusion levels, 250 g kg DM^{-1}. However, Kinely et al. (2016) and Roque et al. (2019) observed anti-methanogenic effects from *Asparagopsis taxiformis* included at 20 and 50 g kg DM^{-1} respectively. Moreover, it has been proven that *Asparagopsis taxiformis* contains an abundance of anti-methanogenic compounds such as bromoform and dibromochloromethane (Machado et al., 2016). Reductions in CH_4 were observed *in vitro* with PT obtained from brown seaweed species (Wang et al., 2009; Vissers et al., 2018). In these two studies, the anti-methanogenic effect of tannins was a consequence of a reduction in OM digestion and the reduced activity of methanogens. However, it is difficult to compare the results of Wang et al. (2009) and Vissers et al. (2018) to this current study and Belanche et al. (2016), as these studies were conducted using the batch culture system over a period of 24 to 72 h, whereas the current study was conducted using the rumen simulation technique over 16 d where the rumen microbial population may have adapted over this period. Previous research suggests that as the protein content of the feed increases, CH_4 production decreases (Johnson & Johnson, 1995; Shibata &

Terada, 2010). Menezes et al. (2016) did not observe any difference in CH_4 production with different CP levels offered to finishing Nellore bulls. However, it should be noted that a small sample size was a limitation in that study which may have had an impact on their results. In the current study, feeding the LP diets resulted in lower CH_4 parameters compared to HP diets. The opposite was observed in this study. Limiting the supply of N in ruminant diets can decrease fibre and OM digestibility (Doreau et al., 1990). Haro et al. (2018) observed that offering low protein diets tended to reduce CH_4 production after 8 h incubation *in vitro*. The reduction in CH_4 emissions associated with feeding the lower level of crude protein may be attributed to a shortage in fermentable protein, as certain cellulolytic bacteria (*R. albus, R. flavefaciens, F. succinogenes,* and *B. fibrisolvens*) protozoa and methanogens are particularly sensitive to a lack of fermentable protein in the rumen (Belanche et al., 2012).

6.6 Conclusions

The reductions in NH_3 observed herein as a result of offering different brown seaweed species may provide the opportunity in shifting the site of N metabolism from the rumen to the small intestine. Feeding brown seaweed species, known to contain PT reduced NH_3 concentrations in both HP and LP diets, but not sufficiently to affect microbial fermentation. There was no negative affect on nutrient digestibility associated with offering brown seaweed species in this study but given the variability in PT content between species and within species ascertaining the level of PT and molecular weight beforehand may help reduce their negative impact on nutrient digestibility. However, further *in vivo* research is required to determine the optimal inclusion level in ruminant diets, which will not impair rumen function.

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Chapter 7

Summary, general discussion and future research

7.1 Summary

Global food security is currently challenged by the increasing demands for food, including meat and milk, which arise through the continuing growth of the world's population and consumption (Searchinger et al., 2019). Concurrently, there are growing concerns as levels of ammonia (NH_3) in the atmosphere continue to rise (Warner et al., 2017) and with excess nitrogen (N) excreted from agriculture (Aneja et al., 2020; Zeng et al., 2018) accounting for 93% of total NH_3 emissions in the EU (EEA, 2020) or 99.2% of total NH_3 emissions in Ireland (Duffy et al., 2019) reductions in N loss from livestock systems are a key requirement and challenge for Irish and European Agriculture. Additionally, N losses into the environment from agriculture have the potential to negatively impact air quality causing respiratory disease due to exposure to high concentrations of fine particulate matter ($PM_{2.5}$), contamination of drinking water, eutrophication of water courses, decreased biodiversity in sensitive ecosystems due to higher concentrations of N, contribute to greenhouse gas (GHG) emissions with increases in nitrous oxide (N_2O), saturation of forest floors with N; and soil acidification through nitrification and leaching (Ndegwa et al., 2008).

Cattle rearing systems in Ireland are predominantly grass-based as 80% of agricultural land is dedicated to grassland (silage, hay and pasture). Feed costs represent the largest single variable cost in beef production in Ireland. Grazed grass is generally the cheapest source of food available for beef (and milk) production provided that the environment and management permit high yields of high quality herbage to be utilised. Over 80% of all farms in Ireland make grass silage (GS) and it accounts for 87% of total grass conserved. The deficiencies in nutrient supply to beef cattle from GS are usually overcome by supplementing with concentrates, which are primarily cereal-based (Drennan et al., 2006). In 2019, 90.6% of the NH_3 emissions from agriculture

was accounted by animal manures, with grazing animals responsible for 10.9% while manure management accounting for 47.4% (EPA, 2020). During the ensiling process, water-soluble carbohydrates are the primary fermentation substrate (Kung Jr, 2001). Therefore, the main carbohydrate substrates available for fermentation in the rumen are slowly fermented fibre substrates, cellulose and hemicellulose, while the N compounds in GS are mainly soluble leading to instant degradation within the rumen (Hersom, 2008). This asynchronous release of energy and N components in the rumen has been considered an important cause of the low N use efficiency for microbial growth observed with diets such as GS (Van Vuuren et al., 1993). The incorporation of cereal grains in concentrate feed formulations can provide an energy source in the form of starch to the rumen microbes, thus allowing a greater capture of N in the rumen (Lardy et al., 2004).

Therefore, the objective in Chapter 3 was to evaluate the effect of supplementing grass silage-based diets with concentrate carbohydrate sources with different fermentation profiles on N metabolism of beef heifers fed to maintenance. In Chapter 4, an in vitro experiment was conducted to evaluate the effects of chitosan differing in molecular weight, at three different inclusion levels on the manipulation of rumen fermentation using artificial Rumen Simulation Technique (RUSITEC). Due to its biodegradability and non-toxic properties, chitosan has received much attention for its diverse applications in medicine and food preservation due to its antimicrobial properties (Kong et al., 2010). To date, its use in ruminant nutrition has not been extensively investigated. In Chapter 5, the chitosan that demonstrated more activity in the RUSITEC experiment in Chapter 4 was selected to evaluate the effects of chitosan inclusion with two levels of dietary crude protein (CP) on nutrient intake and digestibility, ruminal fermentation, and N excretion in beef heifers offered 50: 50 grass

silage concentrate diet. *In vitro* studies can be valuable for screening and informing on the suitability of feeds, substances, compounds for further evaluation *in vivo*, but they do have their limitations (Yáñez-Ruiz et al., 2016). Moreover, previously Goiri et al. (2010) reported that in sheep fed chitosan, ruminal fluid NH_3 concentrations were reduced, while Dias et al. (2017) found that chitosan positively affected dry matter intake, digestibility and increased propionic acid concentrations. Chapter 6 investigated the effects of dietary CP level and brown seaweed source on ruminal fermentation parameters *in vitro* using the rumen simulation technique (RUSITEC). Terrestrial tannins (condensed and hydrolysable) have the ability to reduce the extent of protein degradation in the rumen by direct inhibition of microbial activity and indirectly by forming tannin-protein complexes in the rumen (Patra and Saxena, 2011). Brown seaweeds are the only species able to produce high levels of Phlorotannins (PT) as a result of phloroglucinol polymerisation (Wang et al., 2008). Phlorotannins in *Ascophyllum nodosum* (ASC) have been found to possess strong activity against ruminal microbes (Wang et al., 2006, 2008). The effect of terrestrial tannins has been studied extensively regarding their benefits on ruminant nutrition (Min and Hart, 2003); however, knowledge on the effect of PT on rumen function is still in its infancy.

Nitrogen use efficiency of beef animals is low (10-20%), and the objective of the experiments reported in this thesis were to investigate the effects of dietary strategies to reduce the amount of N lost to the environment in beef production systems.

These experiments were conducted at University College Dublin Lyons Research Farm, Celbridge, Naas, Co. Kildare, Ireland. W23 ENY2 (53^0 17’ 56” N, 6^0 32’ 18” W). The N mitigation strategies were investigated in four experiments, both *in vitro* and *in vivo* and the objectives were to:

1) Investigate the effects of low protein diets, different concentrate carbohydrate sources with different fermentation profiles, and novel feed ingredients on nutrient intake and digestibility, ruminal fermentation, and N excretion in beef heifers offered a grass silage-based diet.

2) Evaluate novel feed ingredients on ruminal fermentation parameters *in vitro* using the rumen simulation technique (RUSITEC).

Chapter 3: Effect of supplementing grass silage-based diets with concentrate carbohydrate sources with different fermentation profiles, on N metabolism of beef heifers fed to maintenance.

In this experiment the objective was to evaluate, the effect of supplementing GS based diets with concentrate carbohydrate sources with different fermentation profiles on N metabolism of beef heifers fed to maintenance. The hypothesis was that offering a carbohydrate that is rapidly degraded within the rumen, will in turn capture more N within the rumen and reduce N excretion.

Nitrogen intake in this study was similar across all treatments with no difference in the amount of N recovered in the urine ($P < 0.05$) at 55% of ingested N, whereas N recovered in the faeces tended to be higher for animals offered MM compared to those offered RB (32% *vs* 28% respectively). The diets in this study were isonitrogenous, balanced in CP with soya bean meal. However, this may have been a limiting factor in the study. While all treatments were balanced for CP, the difference in rumen degradable protein (RDP) between each treatment was not accounted for. Ammonia is the principal source of urea, produced in the rumen from RDP fed to excess or an insufficient energy supply to rumen microbes, metabolised to urea in the liver and excreted in the urine (Colmenero and Broderick, 2006). Therefore, the true potential of each of the three carbohydrates investigated may not have been expressed in their

ability to capture RDP within the rumen. However, urine excretion was unaffected by carbohydrate source and was the major route of N excretion across all dietary treatments (79 g d^{-1}). Reducing urinary N excretion and increasing the portion of ingested N recovered in the faeces is likely to be of benefit to the environment as urinary N is more prone to environmental loss as NH_3 and N_2O than faecal N. The animals offered MM had a higher percentage of N excreted in the faeces coupled with a lower percentage of N excreted in the urine compared to animals offered RB ($P < 0.05$). The animals in this study were fed to maintenance, which is unlikely in a practical situation. Therefore, further research in needed to evaluate the effects of these carbohydrates on N excretion when offered ad libitum. Increases in DMI would lead to an increase in passage rate and possible reductions in digestibility thereby increasing the amount of N partitioned to the faeces.

Chapter 4: Effects of feeding different sources of chitosan, differing in molecular weight, fed at three different feeding levels, on ammonia and methane production in vitro using an artificial RUSITEC.

The objective of this study was to evaluate the effects of chitosan differing in molecular weight, at three different feeding levels on the manipulation of rumen fermentation *in vitro* using the RUSITEC. The hypothesis was that the inclusion of chitosan, low in molecular weight would inhibit ruminal degradation, and in turn reduce ruminal NH_3 concentrations *in vitro*.

Nutrient digestibility (DM, N, and NDF) decreased *in vitro* for all feeding levels and for each molecular weight of chitosan offered. Similar results were observed when chitosan was included *in vitro* (Goiri et al., 2009a; Goiri et al., 2009b; Goiri et al., 2009c; Goiri et al., 2010; Belanche et al., 2016). These decreases in the disappearance of feed from the nylon bags after 48 h indicate that *in vitro* fermentation of the basal

diet may be negatively affected by the addition of chitosan, probably as a result of a decrease in ruminal bacteria and/or in their activity, likely due to the antimicrobial action of chitosan against ruminal microbes (protozoa and fibrolytic bacteria) (Wencelova et al., 2014; Belanche et al., 2016a). However, when offered *in vivo* chitosan inclusion improved DM, CP and NDF digestibility (Araujo et al., 2015; Mingoti et al., 2016; Vendramini et al., 2016; de Pavia et al., 2017). However, caution is required when comparing these studies, as similar results are not generally replicated between *in vitro* and *in vivo* studies. Furthermore, chitosan is a series of compounds with different characteristics and from different sources, all of which influence chitosan as a rumen modulator.

At all inclusion levels, chitosan increased the NH_3 concentrations compared to the control ($P < 0.05$). The increased concentrations NH_3 was likely due to an extra supply of NH_3 from the degradation of amine groups in chitosan and a lower uptake of NH_3 by the rumen microbes, rather than increased proteolysis (Belanche et al., 2016a). Chitosan may have potential as an alternative rumen N source; however, further research is necessary to investigate this without negatively affecting nutrient disappearance.

Chapter 5: Effect of chitosan inclusion and dietary crude protein level on nutrient intake and digestibility, ruminal fermentation, and n excretion in beef heifers offered a GS based diet.

The objective of this study was to evaluate the effects of chitosan inclusion at two dietary CP levels on nutrient intake and digestibility, ruminal fermentation, and N excretion in beef heifers offered 50: 50 grass silage to concentrate ratio. The hypothesis was that offering chitosan to beef cattle would alter N metabolism within the rumen, increase CP digestibility in low CP diets, and in turn reduce N excretion.

The inclusion of chitosan in the diet reduced DM, OM, and CP digestibility ($P < 0.01$), however this did not result in an alteration of DMI. This reduction in nutrient digestibility was likely due to the antimicrobial action of chitosan against ruminal microbes (protozoa and fibrolytic bacteria) (Belanche et al., 2016). Chitosan inclusion had no effect on N intake ($P > 0.05$), however the inclusion of CHI in HP diets did result in an increase in ruminal NH_3 concentrations likely due to an extra supply of NH_3 from the degradation of amine groups in chitosan and a lower uptake of NH_3 by the rumen microbes (Belanche et al., 2016) without additional increases in urinary N excretion. Ruminal NH_3 concentrations in the two LP diets may have been below optimum for microbial and fibre digestion (Kang-Meznarich and Broderick, 1980), however, no differences were observed in ruminal NH_3 concentrations when chitosan was included, which may suggest that chitosan may provide an alternative N, amino acid source in low CP ruminant diets.

Faecal N is primarily of microbial origin, with lesser amounts of undegraded feed protein and endogenous secretions. The increase in faecal N excretion associated with the animals that were offered chitosan is likely associated with the decrease in dry matter, organic matter and CP digestibility. Previously, Mingoti et al. (2016) found that including chitosan in the diet of lactating dairy cows reduced faecal N excretion and they concluded that this was as a result of better utilization of N, which reached the small intestine as a result of changes in rumen fermentation caused by chitosan, correlating with improvements in protein digestibility. However, Goiri et al. (2010) reported a negative impact of chitosan on NDF digestibility in sheep and reduced VFA concentrations in faecal samples, suggesting that chitosan exercised the same antimicrobial action in the cecum as in the rumen, but to a greater extent. Therefore,

further research is required to determine if chitosan can act as an alternative rumen N source and to what level before having any negative effect on nutrient digestibility.

Chitosan inclusion had no effect on urinary N excretion, whereas the higher N intake associated with feeding the higher level of CP, resulted in an increase in urinary excretion for those offered the HP diets. This result supports previous research where the relationship between dietary CP or N intake and N excretion in the urine is far stronger (R^2 = 0.74) than faecal N (R^2 = 0.21) (Mulligan et al., 2004) and strategies that reduce CP in the diet are generally associated with a reduction in urinary N output (Cole et al., 2006; Vasconcelos et al., 2009; da Silva et al., 2016).

The HP diets supplied a higher percentage of RDP compared to those offered the LP diets, resulting in excess N for microbial protein synthesis (Colmenero and Broderick, 2006). In diets where low concentrations of CP are fed, the proportion of urea produced in the liver that is returned to the rumen via blood and saliva increases, resulting in lower proportions of urea excreted via the urine (Reynolds and Kristensen, 2008). Therefore, future work is required to determine the extent to which dietary CP can be reduced in beef cattle diets offered grass silage without affecting performance.

Chapter 6: Effect of dietary crude protein level and brown seaweed source on ruminal fermentation parameters in vitro using the RUSITEC.

The objective of the study was to evaluate the effects of supplementing diets with different CP levels with different brown seaweed species, on the manipulation of rumen fermentation *in vitro* using the RUSITEC. The hypothesis was that the inclusion of brown seaweeds would inhibit ruminal protein degradation and reduce ruminal NH_3 concentrations without affecting rumen fermentation in diets differing in CP content *in vitro*.

All three brown seaweed species offered reduced NH_3 concentrations in overflow vessels ($P < 0.05$), suggesting that the tannins contained within each of the three brown seaweeds investigated was sufficient to form tannin-protein complexes in the rumen (Patra and Saxena, 2011), thereby reducing the extent of protein degradation without affecting nutrient digestion ($P > 0.05$). The feeding of brown seaweeds in this study highlights the potential of brown seaweeds to reduce NH_3 formation in the rumen, which in turn may lead to reductions in urinary N excretion. However, further research both *in vitro* and *in vivo* is required before the feeding of brown seaweeds to livestock is common practice. The level the brown seaweeds offered in this study (50 g kg DM^{-1}), had no effect on nutrient digestibility but given the large variability between species, and other factors such as time of harvesting, habitat, and drying method, a feeding level based on the concentration of tannins within the seaweed may offer an opportunity to feed brown seaweeds without compromising digestibility. Moreover, another point to consider when feeding brown seaweeds, it that brown seaweeds contain high levels of minerals, particularly iodine, and caution is needed to ensure iodine levels are not exceeded when being fed in ruminant diets.

Crude protein level had no effect ($P > 0.05$) on NH_3 concentrations in overflow vessels. In this study, both experimental diets were supplemented with the same energy source, maize meal, and contained similar levels of starch (high protein 30.46% and low protein 31.20%), providing adequate energy for both the low and high protein diets to capture ruminal NH_3 for microbial protein synthesis (Mabjeesh et al., 1997). Additionally, maize starch is more resistant to rumen degradation than other cereal grains, as the starch granules in maize are embedded in the protein matrix, prolamins, which are more resistant to degradation at higher pH (Hoffman et al., 2011), providing

a more sustained energy supply for both the low and high protein diets to capture ruminal NH_3 for microbial protein synthesis.

7.2 General discussion and future research

In light of the new NH_3 reduction targets set out for agriculture by 2030 (DAFM, 2021), research into mitigation strategies has never been more warranted. Agriculture particularly the ruminant livestock sector is facing increased pressure to reduce the amount of N lost to the environment. In this thesis, the effects of dietary manipulations on nitrogen balance and metabolic parameters *in vivo* and the effects of supplementing novel natural feed compounds on ruminal fermentation parameters *in vitro* were investigated.

While ruminant production systems in Ireland are predominantly pasture based, the high levels of NH_3 attributed from the sector are associated with the indoor period. Grass silage is the main conserved forage fed to beef cattle in Ireland. The rate of degradation of non-protein N and soluble protein N in silage within the rumen is high, which is often reflected in a marked peak in ruminal NH_3 concentrations. This rapid and asynchronous release of N relative to carbohydrate is often claimed to be the main cause of low efficiency of microbial protein synthesis with grass silage. A number of approaches have been identified which can influence the degradation of silage N within the rumen, such as silage additives, maturity of the crop at harvest, wilting, and reductions in excessive applications of chemical N on herbage. Therefore, careful consideration is needed in how grass silage is produced. Additionally, plant breeders have a role to develop genotypes with less degradable proteins after the ensiling process.

As the main conserved forage fed to beef cattle in Ireland, grass silage is not sufficient to meet the energy requirements to finish beef cattle for slaughter, with concentrates required offered as an additional energy source. In Chapter 3, N metabolism was evaluated in beef heifers offered carbohydrates with different fermentation profiles in beef heifers offered grass silage fed to maintenance. The results from this study demonstrate that carbohydrates with different fermentation profiles can provide an additional energy source to beef cattle consuming grass silage without affecting N excretion. However, further research is required to establish if the substitution of high-energy feeds such as rolled barley and maize meal with soya hulls in growing and finishing cattle consuming grass silage diets, will not negatively affect performance. Furthermore, there are a number of factors (feeding to maintenance and formulating diets to iso-nitrogenous based on CP concentration) in the study that may have affected the outcome of this study. Feed intake was restricted to maintenance, to avoid excessive weight gain during the study (Zhang et al., 2021), and to ensure their use in future metabolism studies. Furthermore, to ensure that DMI had no influence on N intake due to difference in energy density between the three feed ingredients (Ferreira et al., 2011) in addition to diets formulated to be isonitrogenous (142.6 g d^{-1}). Additionally, daily DMI across all treatments was 6.0 kg DM offered as a TMR at 40: 60 forage: concentrate, and split between two meals at 0800 and 1600 h. With each meal consumed quickly after feeding, it negated the benefit of offering the diet as a TMR and more akin to top-dressing silage with concentrates, verified by the rumen fermentation parameter profiles. The effects of the carbohydrate source may have been greater offered at *ad-libitum* leading to a more synchronous release of energy and N components in the rumen. While all treatments were balanced for CP, the difference in RDP between each treatment was not accounted for. Ammonia is the principal

source of urea, produced in the rumen from RDP fed to excess or an insufficient energy supply to rumen microbes, metabolised to urea in the liver and excreted in the urine (Colmenero and Broderick, 2006). Therefore, the true potential of each of the three carbohydrates investigated may not have been expressed in their ability to capture RDP within the rumen.

The effect of chitosan inclusion was investigated in Chapters 4 and 5 with no impact on N excretion. As chitosan is a series of compounds with different characteristics and from different sources, further studies are necessary to determine if other chitosan forms/sources may have a role in modifying rumen fermentation. In this thesis, the source of chitosan, which had the greatest effect, was of marine origin with high molecular weight and was compared to the chitosan derived from fungal sources with a lower molecular weight. However, the effect of chitosan low in molecular weight of marine origin on the manipulation of rumen fermentation *in vitro* and *in vivo* is yet to be investigated. In addition, further research is required to determine if chitosan has a role as an alternative rumen N source and at what feeding level, without negatively affecting nutrient digestion.

In Chapter 5, feeding the lower level of CP demonstrates that feeding CP in excess to the animal's requirements results in higher levels of N excreted via the urine. Reducing urinary N excretion is more favourable, as the rate of volatilisation of urinary urea N to NH_3 is much faster compared to the organic N compounds in faeces, (Jarvis et al., 1989, Varel et al., 1999). In many feeding situations, farmers continue to offer high levels of CP, in excess of demand, despite the extensive research highlighting no additional performance benefits gained from CP fed to excess, especially to finishing cattle where the CP requirement is much lower than required in growing cattle. Not

only is this a cost to the environment but also to the system in purchasing additional protein.

The inclusion of brown seaweed species in ruminant diets *in vitro* reduced NH_3 concentrations exhibited in Chapter 6. However, knowledge on brown seaweed inclusion in ruminant diets is limited. The variation in the chemical composition of brown seaweeds and between brown seaweed species exists due to factors such as harvest season, light, water temperature and habitat. In this chapter, only three seaweed species were investigated at one inclusion level. These seaweeds were selected based known to have high PT content reported in the literature. If the PT content of the three seaweeds was known before the experiment a smaller screening study could have been explored prior to this study to establish to optimum feeding level for each seaweed. Furthermore, PT and total phenols were the only bioactive analysis conducted, while additional analysis to determine other known bioactives (Laminarin and Fucoidans) may help interpret the results further. Brown seaweeds contain high levels of iodine. Although the levels fed in this experiment did reduce NH_3 without any negative effects on nutrient digestion, it is unclear if these feeding levels in practice would go over the threshold for iodine in ruminant diets. Therefore, further research is warranted into the effects of different sources of brown seaweeds, different inclusion levels of brown seaweeds in diets and finally, this works needs to be done *in vivo* to determine its potential impact on N excretion.

In vitro studies can be valuable for screening and informing on the suitability of feeds, substances, compounds for further evaluation *in vivo* (Yáñez-Ruiz et al., 2016). However, results from *in vitro* studies should be interpreted cautiously as a positive outcome *in vitro* does not guarantee that the identical treatment will have a similar effect *in vivo*. The *in vitro* experiments conducted in this thesis were evaluated *in vitro*

over 16 days and may not have been sufficient to allow the microbial community to adapt to the bioactive compounds within the seaweeds and chitosan. Therefore, the efficacy of feeding brown seaweeds and chitosan will need to be evaluated over longer periods both *in vitro* and *in vivo* to assess any potential microbial adaptation to the bioactive compounds.

Overall, this thesis has demonstrated that reductions in urinary N excretion are achievable by reducing the level of CP fed to beef cattle. Bioactive compounds contained in brown seaweed species have the potential to reduce protein degradation within the rumen, which may lead to reductions in urinary N excretion. Future work is required to determine the extent to which dietary CP can be reduced without affecting performance; does supplementing ruminant diets with chitosan have any role in modifying rumen fermentation; and can the bio-actives in brown seaweeds be characterised and fed in a concentrated form and negate the problems associated with feeding seaweeds.

7.3 Overall conclusions

- Offering a carbohydrate source that is rapidly degraded within the rumen such as rolled barley did not alter ruminal NH_3 concentrations or reduce N excretion in beef heifers offered GS based diets fed to maintenance.
- Similar ruminal NH_3 concentrations were observed across all treatments (rolled barley, maize meal, soya hulls).
- Protein degradation exceeded carbohydrate fermentation 2 h post feeding.

- Supplementing GS based diets with concentrate carbohydrate sources with different fermentation profiles had no effect on N metabolism of beef heifers fed to maintenance**.**

- Ammonia levels increased *in vitro* for all feeding levels and for each molecular weight of chitosan offered.

- Nutrient digestibility (DM, N, NDF) decreased *in vitro* for all feeding levels and for each molecular weight of chitosan offered.

- Increasing protein supplementation in excess of requirements results in increased urinary N excretion, which may have negative environmental consequences.

- Inclusion of chitosan did not reduce N excretion and had a negative effect on nutrient digestibility.

- Feeding the different brown seaweed species used in this study reduced ammonia concentrations *in vitro*.

- Feeding brown seaweeds, known to contain phlorotannins (PT) reduced NH_3 concentrations in low protein diets, but not sufficiently to affect microbial fermentation.

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Chapter 8

Publications

8.1 Thesis publications

8.1.1 Scientific journals

Kirwan, S. F., Pierce, K. M., Serra, E., McDonald, M., Rajauria, G. & Boland, T. M. 2021. Effect of Chitosan Inclusion and Dietary Crude Protein Level on Nutrient Intake and Digestibility, Ruminal Fermentation, and N Excretion in Beef Heifers Offered a Grass Silage Based Diet. *Animals,* 11**,** 771. https://doi.org/10.3390/ani11030771

Kirwan, S.F., Pierce, K.M., Serra, E., Gath, V., Rajauria, G. and Boland, T.M., 2022. Effect of Supplementing Grass Silage-Based Diets with Concentrate Carbohydrate Sources with Different Fermentation Profiles on N Metabolism of Beef Heifers Fed to Maintenance. Ruminants,2(2),pp.188-200.*https://doi.org/10.3390/ruminants2020012*

8.1.2 Conference proceedings and scientific workshops

Kirwan, S., Boland, T., Kelly, A., Serra, E., Rajauria, G. & Pierce, K. 2018. PSXI-35 Effects of chitosan source, molecular weight, and supplementation level on in vitro (RUSITEC) ammonia and methane production. *Journal of Animal Science*, 96**,** 424.

Kirwan, S., Boland, T., Serra, E., Rajauria, G. & Pierce, K. 2019. 413 Effect of chitosan inclusion and dietary crude protein level on rumen fermentation in beef heifers fed a total mixed ration. *Journal of Animal Science,* 97**,** 166.

Kirwan, S., Boland, T.M., Serra, E., & Pierce, K. M. Effect of feeding chitosan with different levels of crude protein on nitrogen intake, digestibility and utilisation in beef heifers fed total mixed ration. 2019. Proceedings of the British Society of Animal Science. April 8-11.

Kirwan, S., Boland, T.M., & Pierce, K. M. Presentation "explanation for high ammonia levels coming from cattle sector, research focus and recent preliminary results. Atmospheric Nitrogen Pollution Workshop: Sources, Impacts and Solutions. University College Dublin 19th May 2017.

Kirwan, S., Boland, T.M., Kelly, A.K., Gath, V., Rajauria, G., & Pierce, K. M. Supplementary carbohydrate source alters rumen nitrogen metabolism of beef heifers. 2017. European Federation of Animal Science Annual Meeting (EAAP) 28th August 28 – 1 September.

Kirwan, S., Pierce, K.M., Condren, S.A., McKay, Z.C., Kelly, A.K., & Boland, T.M. Effect of supplementary carbohydrate source on nitrogen excretion in beef heifers. 2017 RAMIRAN 17th International Conference 4th – 6th September Wexford, Ireland.