The effect of fish silage supplementation on the digestibility of fibre and the nitrogen excretion of sheep fed ryegrass pasture.

Research Report of Experiments with BioMarinus

Conducted by

Kate Smitstra

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Abbreviations

ADF	Acid Detergent Fibre
BW	Body Weight or Liveweight
DM	Dry Matter
DMD	Dry Matter Digestibility
DMI	Dry Matter Intake
FS	BioMarinus Fish Silage
GHG	Greenhouse Gas
LCFA	Long Chain Fatty Acids
N	Nitrogen
NDF	Neutral Detergent Fibre
OMI	Organic Matter Intake
PUFA	Poly-unsaturated Fatty Acids
SCFA	Short Chain Fatty Acids
UDP	Undegraded Rumen Protein

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Introduction

New Zealand dairy systems are pasture-based systems which utilise high quality pasture as the primary feed source for stock. Cows are grazed outdoors, on pasture or forage crops, year-round. This system relies on the seasonal pasture curve, which led to the development of the seasonal calving system, where cows are required to calve within 365 days (d) of the previous calving, in order for their lactation curve to follow a similar trend to the pasture growth curve. This is a low cost and low input system, which relies on maximal intakes of high-quality pasture.

The greenhouse gas (GHG) emissions profile of New Zealand dairy systems is different to that of confinement fed livestock production systems utilised overseas. Due to the reliance on grazing, rather than conserved feeds being delivered to cows housed in barns, enteric methane contributes the largest portion of GHG emissions in New Zealand systems.

In accordance with the pledge by the New Zealand government in 2019 to reduce GHG emissions, New Zealand livestock production systems are under continuous pressure to decrease enteric methane emissions. Furthermore, the government has announced that from 2025, farmers will face a tax for GHG emissions. As methane is the largest contributor to the GHG emissions, reducing methane emissions is a priority. Presently, there are a number of methane inhibitors being developed, but there are currently methane inhibitors on the market which have been proven to reduce methane emissions, or are recognised by government framework to do so. Therefore, there is currently no methane mitigation supplement available to aid farmers in reducing methane emissions, nor is there an opportunity within government policy to reduce the GHG emissions tax by the use of a methane mitigating supplement.

It has been widely acknowledged within the field of ruminant nutrition that the development of a methane inhibitor in pasture-based livestock production systems is highly challenging (Beauchemin *et al*., 2015). Firstly, the typical New Zealand pasture-based dairy system diet delivers a very high-water load to the rumen, coupled with fast rumen fluid passage rates. This means that it is highly challenging to maintain an adequate concentration of any added supplements in this highly transient environment (Saldias 2014). Secondly, in TMR systems, methane inhibitors can be mixed in with the ration, which means that there is even delivery of the inhibitor with the feed intake. Whereas, in New Zealand pasture-based systems, the ability to deliver the inhibitor is generally only available during each milking time when cows consume meal from an in-shed feeding system.

New Zealand has a substantial commercial fishing industry. A waste product from this fishery system is fish silage which consists of the various by-products of preparation for human consumption. Disposing of this waste product is costly, however, this fish silage is able to be utilised as a feed source for ruminants. Fish silage is a good source of protein and energy for ruminants, and fish oil has been extensively reported to inhibit enteric methane production.

A Christchurch company (United Fisheries) developed a novel manufacturing method to produce stable fish silage ('BioMarinus') with a long shelf life (*c.* three months) from fish processing factory by-product, with low energy inputs and the use of naturally occurring proteases to break the by-product down. This would enable widespread use of fish silage across New Zealand.

However, in addition to the unsaturated oils in fish by-product, there is ample protein. Previous reports have described reductions in rumen degradation of forages in the presence of unsaturated oils (Wachira *et al*., 2002; Beauchemin et al., 2020), and as New Zealand pastures are inherently high in CP%, the supplementation of these with fish silage may result in higher nitrogen (N) excretion, which is undesirable.

The objectives of this experiment were therefore to quantify the effect of industry standard fish silage supplementation with BioMarinus on digestibility and N excretion in young growing sheep fed ryegrass diets.

Literature Review

1.1 Introduction

Methane (CH_4) is a major topic in agriculture today due to its significant contribution to global warming, second only to carbon dioxide. The CH_4 accounts for 16% of all global greenhouse gas emissions (Iqbal *et al*., 2008) and can retain 23 times more heat than carbon dioxide which indicates its global warming effects (Soren *et al*., 2015). Due to the structure and function of the digestive tract of ruminants, methane is created as a byproduct of ruminant digestion. Ruminant methane production makes up 15.5% of total atmospheric methane (Mathison *et al.,* 1998).

New Zealand's government has made a commitment to reduce biogenic methane emissions by 24 to 47% below 2017 figures by 2050, including a 10% reduction below 2017 emission figures by 2030 (Ministry for the Environment, 2022). This commitment has been made through the internationally recognized Paris Agreement which has made these commitments a legal obligation for New Zealand to meet. These commitments have put immense pressure on New Zealand livestock production systems to meet these targets in this time frame.

Internationally, there is consistently new research being undertaken into various successful and cost-effective mechanisms in reducing ruminant methane mitigation. There have been a number of successful options developed. However, New Zealand has a unique livestock production system due to having a temperate maritime climate. This allows livestock to graze pasture year-round, creating a very variable and inconsistent rumen environment compared to overseas TMR systems. There is little continuity between international livestock systems and New Zealand livestock systems. This means that methane mitigation options that will work well in international TMR systems, may not have the same effect in New Zealand.

1.2 Methane significance in New Zealand primary industries

1.2.1 Environmental

There is an increasing trend of methane concentrations in the atmosphere. Ethridge *et al.,* (1998) undertook a study that investigated the changes in atmospheric methane from the year 1000 AD to 2000 AD. These measurements were taken from three Antarctic ice cores. Between 1000 AD and 1800 AD, the average global methane mixing ratio was 695 ppb, and varied 40 ppb (Figure 1). From 1800 AD to 2000 AD there was a steady increase in atmospheric methane, reaching 1750 ppb by the year 2000. This trend of increased atmospheric methane over two centuries is caused by human activities, primarily livestock production and rice farming (Iqbal *et al*., 2008).

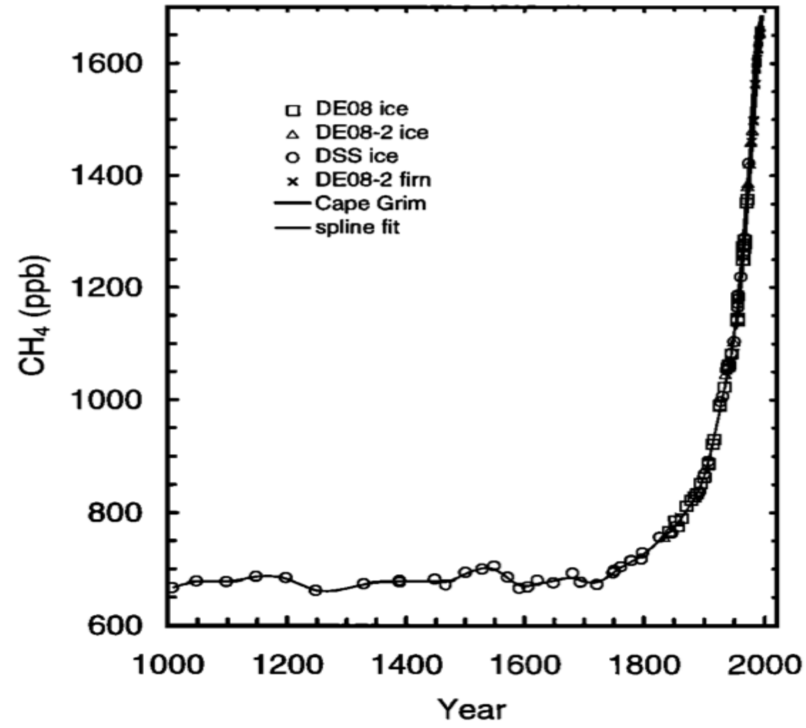


Figure 1: Atmospheric methane concentration (ppb) over the last 1000 years found from the three Antarctic (Law Dome) ice cores corrected for system blank gravitational fractionation (From Ethridge *et al.*, 1998)

Anthropogenic activities have been proven to be the cause of this sharp increase in atmospheric methane over the last 300 years (695 ppb to 1750 ppb). Mathison *et al.* (1998) undertook a study investigating the various sources of atmospheric methane and their quantitative contribution (Table 1). 69.9% of atmospheric methane comes from an anthropogenic source. Almost 20% of total atmospheric methane derived from mining, natural gas and the petroleum industry. Ruminant derived methane contributed 15.5% of total atmospheric methane, second to mining, gas and petroleum in the anthropogenic category. This demonstrates the significant contribution that ruminant animals have on total atmospheric methane.

Table 1: Sources of atmospheric methane and their quantitative contribution (adapted from Mathison *et al*., 1998)

Source	**Tg/year***	**% of total**
Anthropogenic or human related		
Coal mining, natural gas, petroleum industry	100	19.4
Fermentation in animals	80	15.5
Rice paddies	60	11.7
Animal wastes	25	4.9
Domestic sewage treatment	25	4.9
Biomass burning	40	7.8
Landfills	30	5.8
Natural		
Wetlands	115	22.3
Termites	20	3.9
Oceans and fresh water	15	2.9
Others	5	1.0
Total Anthropogenic	360	69.9
Total Natural	155	30.1
Total	515	100.0

1 Tg = 1 x 10^{12} g.

The atmospheric methane contribution by ruminant livestock has significant implications on New Zealand's climate. Global warming is already beginning to show its effects, and this is forecast to continue. The effects include rising mean air and sea temperature, increasing hot extremes, reduced snowfall, changes in intensity and frequency of draught and flood levels, reduction in length and volume of glaciers and sea levels rising (Hopkins *et al*., 2015). These implications of methane emissions and ultimately global warming are significant to multiple aspects of New Zealand's environment and economy.

1.2.2 Economic

Ruminant methane production also has the potential to have an economic significance to New Zealand. More than 21 billion litres of milk are produced in New Zealand annually, and 95% of this is exported (Shadbolt & Apparao, 2016). This is a large portion of our production, however, in 2014, New Zealand's milk only accounted for 3% of global milk production. As a very small player in the global market, New Zealand's products are differentiated by a "clean and green" production image.

A study was undertaken, commissioned by the Ministry for the Environment, and funded by the Contestable Research Fund of the Ministry of Research, Science and Technology to provide an estimate of the value to the New Zealand export trade of our clean green image. The results of this study indicated that the "clean and green' image is worth at least hundreds of millions, possibly billions of dollars, aggregating value elements from dairy, tourism, and organic produce, as well as meat production (Ministry for the Environment, 2001). For the dairy industry in particular, the study showed that if New Zealand's environment was perceived as being degraded, the customers surveyed would purchase 54% less consumer products on average (Ministry for the Environment, 2001). If this lost product could not be redirected, it would equate to an estimated loss of $569 million. This study was undertaken in 2001, so the loss would likely be much greater today.

These results demonstrate the importance for New Zealand production systems to remain ahead of the game when it comes to environmental regulation to maintain our 'clean and green' perception of our international customers. If this is not maintained, New Zealand will suffer drastically economically.

Economically, New Zealand can also suffer from these methane reduction regulations through loss of production. If there are no suitable methane mitigation options found for New Zealand livestock production systems that don't reduce animal production, farmers will be forced to reduce stock numbers to meet the 24 – 47% methane emission reduction by 2050. It is therefore critical that a methane mitigant mechanism is found that will reduce methane while maintaining similar production levels.

1.3 New Zealand dairy production systems

New Zealand's dairy production system has a competitive advantage over most other countries due to its temperate maritime climate (Hopkins *et al*., 2015). This climate allows livestock to be grazed outdoors year-round as feed can be grown through most of the year (Shadbolt & Apparao, 2016). The pastures are made up of predominantly temperate species, based on perennial ryegrasses, legumes, herbs, and annual forage crops such as maize (*Zea mays*) and brassicas (*Brassica* species) (Caradus *et al*., 2021).

Figure 2 below demonstrates the seasonality of New Zealand's pasture production which dictates the annual milk production trends (Shadbolt & Apparao, 2016). Pasture production is largely dependent on climatic conditions, with the highest pasture production occurring in the months of October, November and December, and the lowest pasture production occurring in June, July, and August. In months of feed surpluses, pastures are conserved in the form of silage and hay, which is then fed out in the months of feed deficits.

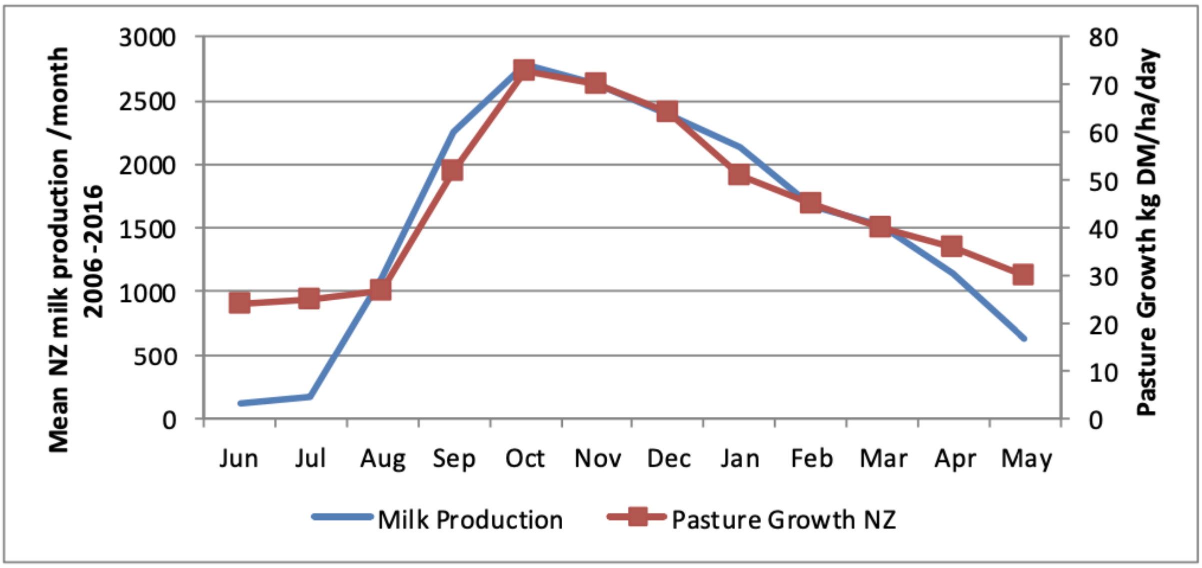


Figure 2: Typical pasture growth curve of New Zealand and mean milk production per month (From Shadbolt & Apparao, 2016).

In months of low pasture production, winter forage species such as fodder beet and kale can also be grown for cows to be break fed on. The seasonality of New Zealand's pasture production creates significant variation of feed intake, diet and therefore milk production throughout the year. Supplying cows with a high DM and energy dense feed throughout the grazing season is a key challenge for New Zealand dairy farmers as this is largely influenced by the season, temperature, water availability and nutrient supply (Wilkinson *et al.,* 2019).

This system reduces the farmers control of diet quality and maintaining a consistent diet for their animals. It also poses challenges for providing consistent supplements to cows, ensuring the specific intake in each mouthful. This unique dairy system in New Zealand also poses challenges for feed access points. With the cows grazing outdoors during the day, and generally coming in to the milking shed twice daily, it only allows farmers two feed access points where they are able to administer certain supplements.

1.3.1 Grazing behaviour

Grazing is described as the consumption and processing of herbage by livestock in the field. The grazing strategy of an animal has a large influence on the pasture intake due to variations in bite mass, bite rate and time spent grazing, as well as the quantity and quality of herbage available.

In New Zealand's livestock grazing systems, there are generally *ad libitum* grazing conditions, which have been described to have between three and five grazing events over 24 h. The three main grazing events take place early in the morning (dawn), mid-day and early evening after milking (dusk) (Gregorini, 2012). This is described as a diurnal grazing behaviour. During dusk there is a longer, more intense grazing period as water soluble carbohydrates (WSC) in the pasture is high and water content is lower (Gregorini *et al.*, 2006).

Studies have shown that as a result of this diurnal grazing behaviour, it also contributes to diurnal patterns in rumen fermentation (de Veth & Kolver, 2001b; Gregorini, Gunter, & Beck, 2008). This results in unusual rumen passage rates throughout the day, making it difficult to keep rumen boluses within the rumen.

1.4 Rumen function

Ruminant livestock have a unique digestive system as they possess a four chambered stomach. The rumen is the largest part of the stomach, which serves as a fermentation "room" (Soren *et al.,* 2015). The rumen has an anaerobic environment which contains many diverse bacteria, protozoa, fungi and archaea which have a symbiotic relationship with the ruminant (Lan & Yang, 2019). This is where fibrous feeds (carbohydrates) are degraded and fermented by microbes with the resulting by-products of fermentation providing an energy substrate for the animal (Soren *et al*., 2015). The internal rumen wall is covered in small projections called papillae which increase the surface area to optimise nutrient absorption.

Through the process of fermentation by rumen microbes, hydrogen (H_2) is produced. The rumen has a normal pH range of 5.5 – 7, therefore the production of hydrogen must be managed to maintain this pH range (Hungate, 2013). If the production of hydrogen exceeds the capacity of the rumen for removal, the rumen pH may decline. This may result in microorganisms involved in the fermentation process being unable to function optimally and sustain the complete oxidation of substrates (Sharp *et al.,* 1998).

1.4.1 Carbohydrate digestion

The primary component of pasture DM, as well as the main source of energy for grazing animals comes from the carbohydrate portion of the feed. Carbohydrates are made up of carbon, hydrogen and oxygen, and have the chemical formula (CH_2O). There are two primary forms of carbohydrates, structural carbohydrates (SC) (e.g. fibre, cellulose, hemicellulose, lignin and pectin) and non-structural carbohydrates (NSC) (e.g. water soluble carbohydrates and fructose's). Ruminants are able to digest a wider range of carbohydrates than most other mammals (Sutton, 1971). New Zealand's pastures contain higher concentrations of NSC than tropical pastures, which are able to be rapidly fermented by rumen microbes, providing a substantial energy source for microbes. Approximately 90% of carbohydrate digestion is done through fermentation to short chain fatty acids (SCFA) (Sutton, 1971)

The first form of digestion that the carbohydrates go through is an anaerobic process in the rumen. Dietary carbohydrates are degraded to hexoses and pentoses (Figure 3). Fermentation then takes place by the rumen bacteria and protozoa which results in the production of volatile fatty acids (propionate, butyrate and acetate). Ruminants fed high fibre forage diets encourage the growth of acetate producing bacterial species, creating typical acetate: propionate: butyrate molar proportions in the region of 70:20:10 (Bannink & Tamminga, 2006). Starch rich diets favour the production of propionate producing bacteria, increasing the proportion of propionate at the expense of acetate (Bannink & Tamminga, 2006). This is a result of the different forms of carbohydrates being fermented by different species of the rumen microbial population.

Volatile fatty acids (VFA's) are then available to be absorbed through the rumen wall, serving as energy sources for the ruminant. Through the process of fermentation into VFA's, hydrogen is created as an intermediate from the reducing equivalents released from glycolysis and pyruvate oxidative decarboxylation to acetyl-CoA (Lourenco *et al.*, 2010). Hydrogen will always be present in the rumen, and these concentrations need to be managed to maintain a stable rumen environment.

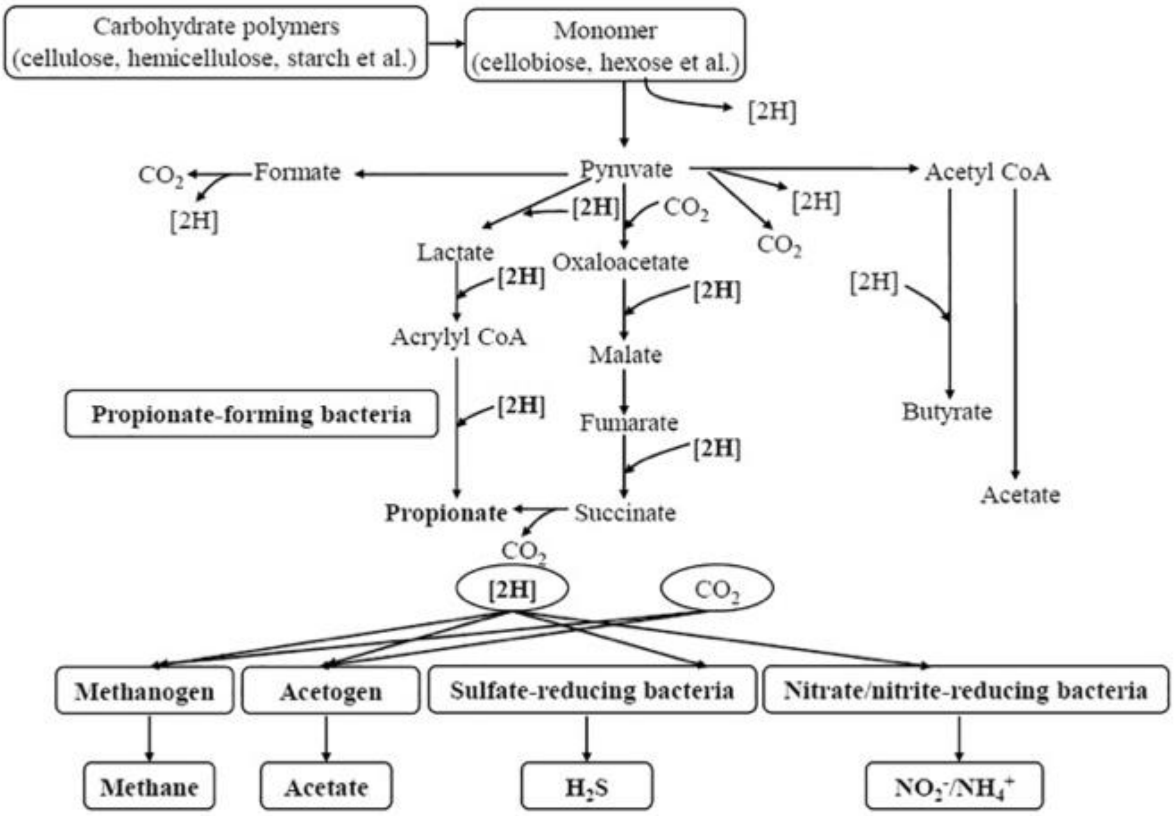


Figure 3: Feed fermentation and hydrogen disposal routes in the rumen (From Lan & Yang, 2019)

1.4.2 Metabolisable protein supply

The microbial processes of the rumen provide the ability for ruminants to convert fibrous and low quality feed, into essential nutrients for the ruminant (Dewhurst *et al*., 2000). Rumen microbes have the unique ability to utilise non-protein forms of N, such as ammonia, to synthesis the full spectrum of amino acids, as opposed to mammals who rely on essential amino acids from the diet. Two thirds to three quarters of the amino acids absorbed by ruminants derives from microbial protein, so it is an essential protein source (Dewhurst *et al*., 2000).

Feed enters the rumen, containing protein in the form of peptide chains, amino acids, or non-protein N forms such as ammonia and urea. The structure of the protein is a determining factor to its degradability as it affects the susceptibility to microbial proteases (Bach *et al*., 2005). Once feeds enter the rumen, bacteria attach to the feed particles, which allows for the activity of cell-bound microbial proteases to begin degradation of the feed particle. Through this process, the protein is degraded into peptides, amino acids or ammonia, which are then transported inside microbial cells (Bach *et al*., 2005). Once inside the microbial cell, if sufficient energy (ATP) is available, the nitrogen containing compound will be used as a substrate for microbial protein synthesis. This mechanism allows the microbial cells to replicate, so long as sufficient nutrients are supplied to the microbes. Microbes may then die, and be degraded, with their own cells becoming substrate for other microbes to grow. The microbes, pass through to the small intestine, are degraded and absorbed. This is referred to as the microbial protein component and serves to satisfy the metabolizable protein requirement for the ruminant.

Due to New Zealand's pasture based dairy systems generally containing excess concentrations of dietary N, not all of the N consumed in the diet can be utilised for microbial protein production and animal production (Pacheco & Waghorn, 2008). The surplus N will be absorbed as ammonia through the rumen wall and either recycled back to the rumen through salvia or excreted out of the body in the urine. (Tandon & Saddique, 2016).

There are multiple factors that affect the ruminal protein degradation, including rumen pH, oxygen concentrations, rumen outflow rates, nitrogen compound, vitamins and minerals, level and type of feed (Harun, 2019).

Protein solubility can vary, therefore degradability in the rumen is variable (Chalupa, 1975). If the protein is undegradable by the microbes, it leaves the rumen as bypass protein or undegradable protein (UDP). This means that when it arrives in the small intestine the feed has the same amino acid profile as when it entered the rumen, whereas protein entering the small intestine as microbial protein has a different amino acid profile to the feed protein that was ingested. Because UDP is not degraded in the rumen, it cannot leave the system as ammonia, and therefore, often has better utilisation.

1.4.3 Biohydrogenation

The use of dietary fat supplements has increased as farmers are striving for supplements with increased energy density. Dietary fat has an alternative digestion mechanism than protein and carbohydrates. Furthermore, the two forms of fat, saturated and un-saturated fats have different pathways of metabolism in the rumen (Lock *et al*., 2006). Unsaturated fatty acids (UFA) have a toxic effect to rumen microbial bacteria. When UFA enter the rumen, they must therefore undergo biohydrogenation to become saturated fatty acids (SFA). Biohydrogenation is the process in which the double bonds of unsaturated fatty acids are broken, adding hydrogen to the compound, to create saturated fatty acids. This means that surplus hydrogen from the rumen environment is used in the biohydrogenation process, competing with methanogens, which reduces the amount of hydrogen available for methane production.

1.4.4 Methane production

Hydrogen levels in the rumen must be managed to maintain a stable environment in the rumen and prevent feedback inhibition (Patra *et al.,* 2017). The rumen is unable to function without hydrogen being expelled. Hydrogen is transformed into dihydrogen (H_2) in the rumen, which is present in two forms, dissolved H_2 (dH_2) and gaseous (gH_2) (Beauchemin *et al*., 2020). Dissolved H_2 does not accumulate in the rumen because it is transferred from the fermentative network of bacteria, protozoa and fungi to the methanogenic archaea (Beauchemin *et al.,* 2020). Rumen methanogens have a close relationship with the ruminal protozoa, bacteria, and fungi, through this interspecies H_2 transfer (Patra *et al*., 2008). The unique environment of the rumen, in which there is readily available carbon dioxide (CO_2) and hydrogen (H_2), along with a rapid passage rate, creates a suitable environment for a community of archaea (Patra *et al*., 2017).

Methanogenic archaea are a group of strictly anaerobic Euryarchaeotic (Thauer *et al*., 2008). They live freely in the rumen liquid or as members of the biofilm, attaching to food particles. The majority of these archaea are hydrogenotrophic rather than acetolactic, meaning they are able to metabolise molecular hydrogen as a source of energy instead of acetic acid (Patra *et al*., 2017). The energy metabolism of these archaea is restricted to the formation of methane from CO_2 and H_2, formate, methanol, methylamines and/or acetate (Thauer *et al*., 2008). Rumen methanogens gather up H_2 and CO_2 substrates and synthesize them to create methane (CH_4) through a process called methanogenesis. Methane is regarded as the major disposal route of fermentation hydrogen (Bannink & Tamminga, 2006; Beauchemin *et al*., 2020).

Figure 4 below demonstrates the seven step pathway of methanogenesis. Methanogenesis involves the reduction of CO_2 to CH_4, using H_2 and a number of reduction co-factors as electron donors (Goldman *et al*., 2009).

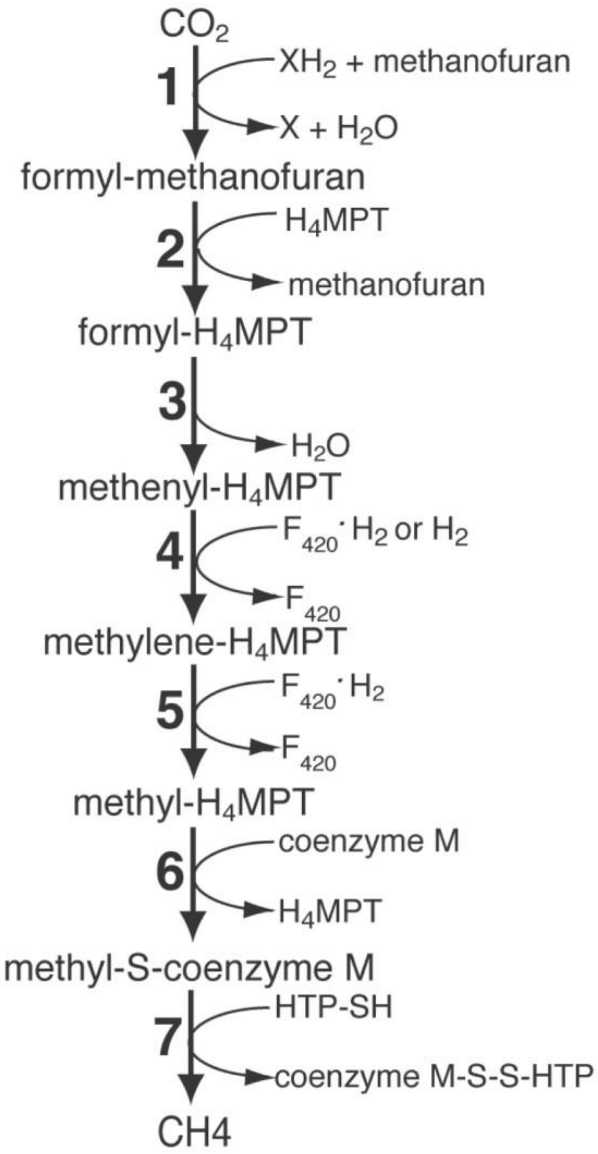


Figure 4: The hydrogenotrophic methanogenesis pathway of class I methanogens (From Goldman *et al.,* 2009)

1.5 Factors effecting ruminant methane production

1.5.1 Dry matter intake

Methane production has been proven to be directly influenced by DMI. A study undertaken by Molano and Clark (2008) investigated the relationship between dry matter intake (DMI) (kg) and methane emissions (g/ d). Sixteen lambs were used in this trial who were randomly allocated into groups, fed at four different levels of allowance. These were 0.75, 1.0, 1.25, and 2.0 times their maintenance level. After 5 d of feeding at this level, the methane emissions were measured daily for a 5 d period. The following 10 d was a repeat of this trial; however, they were fed ryegrass that was at a reproductive stage. There was a strong positive correlation between DMI and methane emissions (r^2 = 0.83) (Figure 5). Similar findings were reported by Shibata *et al.,* (2010). These results can be explained by an increasing DMI resulting in more organic matter fermentation in the rumen. As hydrogen production is directly proportional to the amount of feed fermented, increasing DMI increases production of hydrogen. These hydrogens must then be disposed of to maintain the rumen pH within the physiological optimum, which encourages methane production, as does an increased fractional passage rate of solids and liquids.

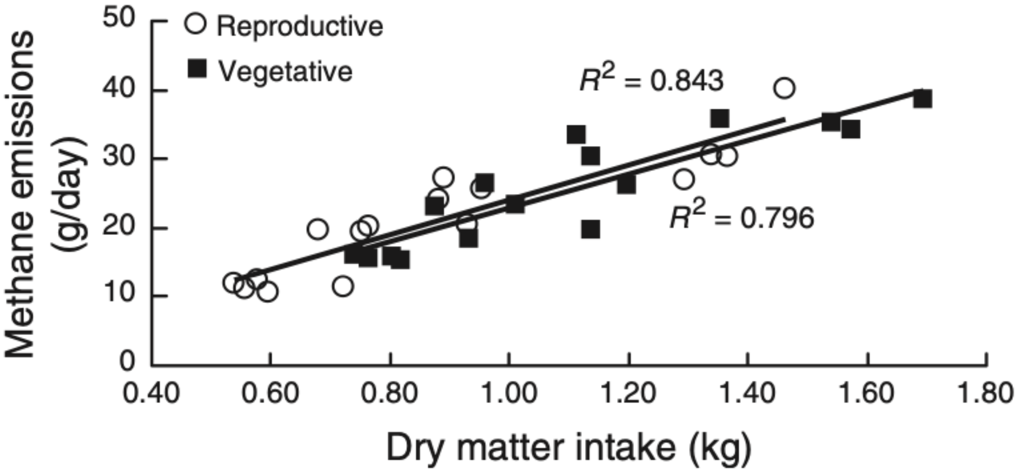


Figure 5: The relationship between daily CH4 emissions and DMI for reproductive and vegetative stage of growth of the ryegrass (From: Molano and Clark, 2008)

1.5.2 Carbohydrate digestion

Carbohydrate sources in ruminant diets also have an influence on methane emissions. Studies have shown that ruminants that have access to concentrate rich diets such as cereals and grains emit less methane than those on forage-based diets (Chandramoni *et al*., 1999; Shibata & Terada., 2010; Benchaar *et al.,* 2001). Benchaar *et al.* (2001) undertook a study investigating the relationship between forage/concentrate ratio and methane emissions. The results showed an increase in methane from 2.55 to 2.70 mega-calories (Mcal) d^{-1} when the concentrate in the diet increased from 0 to 20% (Table 2). A further increase in concentrate to 50% and 70% saw a decrease in total methane produced to 2.61 and 2.12 Mcal d^{-1} respectively. The methane production per % of gross energy increased linearly with increasing concentrate levels from 4.98 Mcal d^{-1} at 0% concentrate to 3.46 Mcal d^{-1} at 70% concentrate. The methane production per % of DMI also decreased with increasing concentrate levels from 7.99 Mcal d^{-1} at 0% concentrate to 4.87 Mcal d^{-1} at 70% concentrate.

Table 2: The effect of forage/concentrate ratio on rumen fermentation and methane production (From Benchaar *et al*., 2001)

	Forage/concentrate ratio			
Intake	**100:0**	**80:20**	**50:50**	**30:70**
DM (kg d^{-1})	15.00	15.00	15.00	15.00
OM (kg d^{-1})	10.01	10.95	11.68	12.18
Starch (kg d^{-1})	-	1.02	2.55	3.58
NDF (kg d^{-1})	6.36	5.57	4.02	2.99
GE (Mcal d^{-1})	51.15	55.91	59.02	61.09
Passage rates				
Liquids	3.42	3.07	2.70	2.55
Solids	1.31	1.22	1.11	1.08
Methane production				
Mcal d^{-1}	2.55	2.70	2.61	2.12
% GE intake	4.98	4.83	4.43	3.46
% DE	7.99	7.28	6.30	4.87

Digestion in the rumen is largely dependent on microorganisms which need energy, nitrogen, and minerals to function. These are obtained from the diet consumed. The quality of the forage that has been consumed therefore affects the activity of the rumen microbes. Concentrates contain readily fermentable sources of carbohydrates. When consumed, these cause an increased rate of fermentation and shifts the fermentation pathway towards increased propionate production. The production of propionate creates a competitive pathway to methanogenesis for H_2 use in the rumen (Wang *et al*., 2018). Therefore, there is a reduction in methane production due to a decrease in H_2 availability.

This is relevant to New Zealand pasture based dairy production systems as studies have shown that highly managed pastures tend to have higher sugar contents, which is the traditional dairy diet in New Zealand (De Veth & Kolver, 2001)

Highly soluble carbohydrates also result in a decreased ruminal pH which decreases the methanogen activity and can kill rumen protozoa. This also destroys the habitat of around 20% of methanogens (Stum *et al*., 1982)

1.5.3 Production status

Production status of a ruminant (e.g., how much milk it is producing) is influential on the amount of methane that it produces. As production increases, DMI increases which increases the amount of substrate in the rumen. Studies have reported that at peak lactation, a cow can emit 430 g methane/ d and it can decrease to 250 g methane/ d as the milk yield decreases (Cottle *et al.,* 2011). The major contributor to this decrease would be the decreased DMI of the animal.

Although total DMI increases methane output, higher quality feed, due to different carbohydrate structures, will decrease output per unit of DMI, therefore increasing efficiency.

1.5.4 Environmental temperature

Environmental temperature has been proven to be an influential factor on methane production and its production rate (Shibata & Terada., 2010). Studies have found that in a tropical environment, the contents of the cell wall, acid detergent fiber and lignin increase, resulting in the feed becoming less digestible. This increases the amount of methane produced per unit of product as there is a decrease in efficiency of animal production (Shibata & Terada., 2010).

In contrast, Moss *et al*., (2001) found that methane output increased at the lower temperature of 5°C compared to 24°C. This was explained by the increased DMI at 5°C, increasing substrate availability in the rumen, therefore causing increased microbial activity and methane production. Similarly, Kurihara *et al*. (1995) reported a reduction in CH_4 emissions as temperature increased because of decreased feed intake. CH_4 production per DMI however increased with higher temperatures because of higher feed retention time, therefore a lower outflow rate of methanogens from the rumen to the abomasum (Kurihara *et al.,* 1995). From these findings, Kurihara *et al.* (1995) established a significant regression equation between DMI and CH_4 production at 18℃ and 30-32℃ respectively. It was concluded that CH_4 production per DMI increased at high temperatures and was around 10% higher at temperatures above 26℃ than at 18℃ in cows being fed at maintenance level.

1.5.5 Biological factors

Protozoal-methanogen interaction

Studies have demonstrated that protozoa in the rumen can contribute nearly 37% of the rumen methane emissions (Finlay *et al*., 1994). It has also been demonstrated from further studies that rumen fluid with a higher number of protozoa generally have a higher rate of methane production (Klieve & Hegarty, 1999). These effects are a result of the symbiotic relationship between protozoa and methanogens in the rumen. Protozoa can rapidly ferment soluble sugars which creates a ready source of H_2 for methanogens.

The protozoal – methanogen interaction does play a role in rumen methanogenesis, therefore any effort to decrease rumen methane production must be considered in conjunction with the effect it will have on ruminal digestibility which correlated with production performance (Benepal, 2012).

1.6 Methane mitigation options

1.6.1 Diet manipulation – forage quality

Mitigating methane through diet manipulation can be done in two ways: improving forage quality and changing the proportions of the diet. The quality of the forage influencing methane output through altering the fermentation process within the rumen. Consuming high quality forages also increases DMI in animals which is associated with an increased rumen passage rate, and decreased CH_4 per gram of DMI (Beauchemin *et al*., 2020). Altering the amount of rapidly fermentable carbohydrates in the diet, and less NDF, makes the feed more digestible and therefore has a higher passage rate through the rumen (Haque, 2018). Animals fed high-quality forage diets are also more productive which results in a lower CH_4 emission. In New Zealand's outdoor grazing system this strategy can be implemented through ensuring optimum grazing management, timing of harvest for conserved feed and feeding forages with superior digestibility.

There is a second opinion to this strategy as differences in feed quality don't always alter CH_4 emissions (g/ d) as high quality forages have a greater ratio of non-fiber carbohydrates to NDF and less lignified NDF which promoted organic matter degradation in the rumen. This increases the amount of hydrogen available for methanogenesis, therefore increasing absolute CH_4 production due to greater DM digestion (Beauchemin *et al*., 2020).

1.6.2 Diet manipulation – concentrates

Concentrate diets are associated with lower CH_4 production (g/ kg DMI) (Johnson & Johnson, 1995). This is a result of the starch fermentation process creating more propionate and butyrate than cellulose in forage diets thus competes with methanogens for hydrogen, reducing the amount of methane produced. The consumption of high starch diets can also reduce the ruminal pH which inhibits the growth of methanogens. This can, in turn, reduce the amount of fiber digestibility occurring and therefore pose a risk for acidosis (Beauchemin *et al*., 2020). Compared to cellulose digestion, starch has a faster rate of digestion which results in elevated dH_2.

A study undertaken by Mathison et al. (1998) compared three different diets fed to 350 kg steers to investigate the effect it would have on methane emissions. One group was offered poor quality pasture at 5 kg/ d DM, another group was fed good quality pasture at 9.9 kg DM and the last group was fed a high concentrate diet at 9 kg of DM. Daily live weight gains were 0.25, 1.1 and 1.5 kg/ d respectively. CH_4 production was 7, 6 and 4% of gross energy intake which was expected to be around 115, 195 and 119 g/ d respectively (460, 180 and 80 g/ kg). Animals fed the higher concentrate diet would also reach slaughter weight sooner and therefore reduce overall emissions from the animal.

Increasing concentrate based diets as a strategy to reduce methane comes with challenges such as the global capacity to grow more grains is limited. Concentrate production also emits other GHG which are not taken into consideration.

1.6.3 Improving genetic merit

Improving the genetic merit of a herd by investing in more productive stock can reduce methane emissions by 20 – 30% through reducing herd numbers and maintaining the same production. This is a result of ruminants improving their ability to partition nutrients into product preferentially to maintenance and/or growth which has increased animal efficiency. Kirchgessner *et al.* (1995) suggested increased milk production from 5000 litres annually to 10,000 litres only increased methane production by 5% (110 to 135 kg methane annually).

1.6.4 Genetics

There is continuous research taking place on the heritability traits of methane production, to breed lower emitting ruminants. Recent findings have shown CH_4 production on a g CH_4/ d basis have a moderate heritability of 0.29 and 0.40 in sheep and cattle respectively (Pickering *et al*., 2015). On a yield basis however (g CH_4/ kg DMI), this is much lower at 0.13 and 0.19 respectively (Pickering *et al.,* 2015)

Reducing methane emissions through genetic selection programs poses a major challenge due to the difficulty of measuring methane emissions in a manner that represents the long term methane emission phenotype of the animal (Beauchemin *et al.,* 2020). Because methane production is predominantly driven by DMI and rumen fermentation, emissions fluctuate depending on the status of the animal diet and time of feeding. Therefore, obtaining accurate records of low-cost estimates of ruminant emissions under commercial conditions is challenging.

1.6.5 Defaunation

Defaunation is another strategy that has been used to reduce methane production, which is done through reducing the number of ciliate protozoa in the rumen (Moss *et* al., 2000). Rumen protozoa are important, but not essential in the rumen environment. Methanogens have been observed to be attached to the exterior of rumen protozoa. Newbold *et al.* (1995) observed that methanogens associated with ciliate protozoa were responsible for 9 to 25% of methanogenesis in the rumen fluid. Rumen protozoa are also large producers of hydrogen in the rumen which in turn creates more methane.

There are multiple techniques of defaunation that have been tested, however none of these have been able to be used routinely due to toxicity problems to either the rumen microbial population of the host animal (Benepal, 2012). There has been recent research undertaken into using plant secondary metabolites as defaunation agents, particularly saponin containing plants. Saponins are glycosides which are said to interact with cholesterol in eukaryotic membranes but not in prokaryote membranes (Tan *et al*., 2020). A study undertaken infusing sheep with pure saponin showed a decrease in rumen protozoal numbers. However, because ciliate protozoa play an active role in fibre breakdown in the rumen, defaunation has been shown to adversely impact fibre breakdown (Bhatta, 2015). Therefore, the use of defaunation agents to reduce methane needs to be balanced against these effects.

1.6.6 Vaccinations

Currently, there is a lot of investigation into the possibility of a methane mitigating vaccination. A vaccination with long term effects on methanogenesis would be beneficial for pasture based systems where intake, diets and rumen function fluctuates so often during the day. The concept of a methane mitigating vaccination is based on the vaccine inducing antibodies in saliva which, when entering the rumen, would suppress the growth of methanogens (Subharat *et al*., 2016). An almost 8% reduction in methane production in sheep who were vaccinated against three methanogens was reported by Wright *et al.,* (2004). However, for various reasons, a practical and successful vaccination against methanogens has not yet been achieved (Martin *et al*., 2010), and probably never will be.

1.6.7 Feed additives and supplements

Lipids

Various studies have demonstrated the opportunity of lipid supplementation in decreasing enteric methane production (Grainger & Beauchemin, 2011; Grainger *et al*., 2008) by up to 50% (Machmuller, 2006). There are various mechanisms as to which lipids can reduce methane production.

As mentioned earlier, lipids are not able to be fermented by rumen microbes. Therefore, lipids must undergo biohydrogenation which is the process in which the double bonds of unsaturated fatty acids are broken, adding hydrogen to the compound, to create saturated fatty acids. The biohydrogenation process therefore competes for hydrogen with methanogens, reducing the amount of hydrogen available for methanogenesis, in turn reducing methane emissions (Johnson & Johnson, 1995).

Another mechanism by which fat supplement can reduce enteric methane production is through the encouragement of propionate production (Harventine & Allen, 2006). Propionate is one of the major VFA's produced that enter the blood stream and serve as an energy source for ruminants. Propionate production in the rumen requires hydrogen. Therefore, the increased production of propionate in the rumen results in increased demand for hydrogen and therefore reduced hydrogen available for methane production (Wang *et al*., 2003).

Aside from methane reduction, lipid supplements also have other benefits for feeding, including increasing the energy density of feeds, which is generally the reason for the addition of fat supplements to ruminants diet (Benepal, 2012). Fat supplements are also generally of natural origins which is beneficial for today's livestock production system trends. They also have the potential to change the fatty acid profiles of meat and milk, increasing the efficiency of fat soluble nutrients. The supplementation of fat can also reduce the dustiness of the feed and enhance feed flavour (Toprak, 2015). For these reasons, the use of fats and oil supplements to reduce methane production is preferred over other approaches (Beauchemin *et al.,* 2020).

It has however been speculated that when ingested in large quantities, fat supplements can however have a toxic effect on rumen protozoa which are essential for fibre digestion (Rassmussen & Harrison, 2011), thus, reducing fibre digestion in the rumen and therefore DMI (Allen, 2000). More recent research however has challenged these findings by showing no negative DMI and NDF digestibility when fed supplements high in fat (Costa *et al*., 2017).

Bovaer

Recent research has been undertaken investigating the potential of 3-nitrooxypropanol (3-NOP) as a methane mitigant (Alemu *et al*., 2021), 'Bovaer' marketed by DSM-Firmenich (Copenhagen, Demark).

The Bovaer reduces methanogenesis in the rumen through inactivating the enzyme methyl-coenzyme M reductase (MCR) which is used by rumen archaea. The MCR catalyses the final step in methanogenesis so this slows down the rate of methane production (Alemu *et al*., 2021). It is fed as a granular supplement, and must be fed daily.

Studies undertaken in TMR diets overseas have recorded methane reductions of between 26 and 39% (Alemu *et al.,* 2021; Moreno *et al*., 2019) using Bovaer. TMR diets allow for the supplement to be mixed through the diet thoroughly and for Bovaer to be present in every mouthful. Due to the nature of New Zealand pastoral based livestock systems, incorporating a methane mitigant supplement that needs to be mixed thoroughly through the days diets is impossible.

Most New Zealand dairy systems are able to feed supplements through the milking shed twice daily, but unable to feed supplement evenly in the paddock during grazing. A recent unpublished study undertaken by Muetzel et al., (2019) investigated the potential of Bovaer to be fed in pastoral farming systems. When fed at a rate of 2.5 g/ d per cow during milking, and access to pasture was withheld for 1 h to simulate the delay in time from milking to grazing, there was no significant effect of Bovaer on methane detected. These results demonstrated the challenge of Bovaer incorporation in a traditional New Zealand pastoral dairy system, where rumen fluid passage rates are higher than TMR feeding systems used internationally (Saldias, 2014).

1.7 Potential mitigation options in New Zealand

Although there are numerous successful methane mitigation options internationally, as well as more currently being investigated, due to the uniqueness of New Zealand production systems, these are not always suitable to be used successfully in New Zealand. For example, while reported as effective overseas, Bovaer has not seen successful methane reductions in high intake pastoral based systems common in New Zealand (Muetzel *et al*., 2021).

Decreasing methane production through genetics is an option for New Zealand farmers, however, as mentioned earlier, this mechanism is still very much in the development stages. It will also take a number of generations of replacement cows for it to come into effect, successfully reducing the whole herds methane production. Farmers mind set will also have to be changed in terms of choosing genetics to reduce methane rather than increasing their herds breeding and production worth.

Many New Zealand farmers run a low cost pasture based system, where supplements aren’t fed, or those that are being fed are silage, or the grazing of a fodder crop. Increasing the concentrate supplementation in New Zealand pasture based diets is an expensive task as grain prices continually increase and the availability of grain in New Zealand decreases.

For a New Zealand pasture based system, lipid supplementation has the greatest potential. Fats can be sourced naturally from local sources, minimising the environmental footprint. Lipids are able to decrease methane production in high rumen passage rate, pasture based diets.

1.7.1 Fish silage opportunity

Fish silage is a liquid product that is produced from the by-products of fish processing for human consumption. The components of the fish that aren't used for human consumption, including the offal and bones, are ground up and ensiled (Arruda *et al.,* 2007).

New Zealand's aquaculture industry is growing, with the industry primed for development, and is anticipating increases in export earnings to NZ$ 3 billion by 2035 (Stenton-Dozey *et al.,* 2020). To achieve this target, the industry must maintain its clean and green image to keep customers satisfied. Some major fisheries in New Zealand, such as United Fisheries, are ahead of the game in this task by utilizing their fish waste to create fish silage using a low energy, low input approach.

This is done through cold processing where papain proteolytic enzymes are added in an anaerobic environment to preferentially break down protein structures to simple peptides or amino acids in a short and intense period. Across seasons and years, the end product has been demonstrated to consistently have approximately 45-60% protein content, 20-30% oil content and 10% mineral content (SJ Gibbs *pers comm*.)

BioMarinus fish silage is a natural product, produced from by-products of New Zealand fisheries by United Fisheries, Canterbury, New Zealand. This makes it a very environmentally friendly product, backing a clean and green image. With a high oil content, it has the potential to be used in New Zealand as a methane mitigant for ruminants. As a secondary benefit, fish silage would also increase PUFA supply to the ruminant, as well as supplying high quality diet protein.

Previous Lincoln University studies have demonstrated that BioMarinus fish silage reduced enteric methane production (Gibbs 2010), however, being a lipid supplement, it is important to quantify the effect that this dose would have on DMI, DMD, DOMD and NDF DMD when used as a methane mitigant. With a high CP concentration, it is also important to quantify the effect on N utilization and efficiency.

1.8 Conclusion

Methane reduction needs to be achieved for New Zealand's livestock production system in order to meet the government's goal of a 24–47% reduction by 2050. Internationally, various mechanisms have been investigated to reduce ruminant methane, however, due to New Zealand's unique livestock production systems and the effects this has on rumen function, these have been posed challenging to implement successfully in these systems.

Lipids have been found to have the greatest potential for New Zealand's pasture based system as fats can be sourced naturally from local sources, minimising the environmental footprint. Lipids are able to decrease methane production in high rumen passage rate, pasture based diets. In New Zealand, fish silage is a lipid supplement that has been proven to reduce methane production in pasture based diets, and has presented an opportunity for livestock production systems in New Zealand.

Although fish silage has been demonstrated to reduce enteric methane outputs, no research has been undertaken on the effect of fish silage supplementation on DMI, DMD, DOMD and NDF DMD. With previous studies having shown that lipids can reduce NDF digestibility in TMR diets, it is important that this is investigated in New Zealand pasture-based diets to demonstrate to farmers it will not reduce production. With fish silage containing high CP, it is also necessary that the effect of fish silage supplementation on N excretion is also quantified to ensure there is no increase when fed.

The objectives of this study are therefore to investigate and quantify the effect that fish silage (BioMarinus) supplementation for methane reduction will have on DMI, DMD, DOMD, NDF DMD and N excretion in a traditional pasture based livestock system in New Zealand.

Materials and Methods

In order to meet the objectives stated above, requiring a close approximation of the mitigation of methane in New Zealand pastoral livestock industries, it was necessary to replicate the diets as closely as possible. Therefore, the study used fresh harvested, highly managed, ryegrass dominant pastures, to mimic the rumen environment and high rumen passage rates of typical New Zealand ruminant production systems.

The study also used unrestricted intakes in a metabolism crate pen feeding system, with an experimental design of a cross over using two groups of eight growing sheep with total urine and faecal collection, in order to achieve high DMI, closely quantify digestibilities and N excretion, and have appropriate statistical power to determine small changes in the desired parameters.

The study also used direct drenching of determined doses with fresh fish silage, rather than drying the product for inclusion in a prepared product such as a pellet, where some uncertainty on the effect of preparation on the silage would result.

1.1 Location

This experiment was conducted at the Johnstone Memorial Lab Research Farm at Lincoln University, Canterbury, New Zealand (-43.64, 172.45). This trial was approved by the Lincoln University animal ethics committee in accord with the NZ Animal Welfare Act 1999, prior to commencement.

1.1.1 Experimental design

This experiment was a repeated measures cross-over design. It was undertaken in two separate runs (1 and 2), where two groups of eight similar entire male lambs from a single bloodline were studied in each run. During Runs 1 and 2, all lambs were fed a harvested ryegrass diet at unrestricted intakes. Group 1 received a daily 60 ml fish silage drench. This was administered at the 10 am daily, prior to their daily feed allocation. These diets were fed for 14 d in separate pens in the wool shed, before being transferred to metabolism crates.

After returning to the woolshed following Run 1 in the crates, group 1 and group 2 switched diets with group 2 now being supplemented with 60 ml daily drench of fish silage and both still being fed ryegrass for 14 d in the separate pens in the wool shed, before being placed back into the metabolism crates for 7 d.

1.2 Fish silage

Fish silage (BioMarinus) was produced by and sourced from United Fisheries Ltd in Sockburn, Christchurch. This product was manufactured through the enzymatic hydrolysis of fish, utilising the by-products from the United Fisheries fish processing plant. These fish are supplied from a Sustainable Fishing Resource and harvested according to the New Zealand Quota Management System. During the experiment, the fish silage was stored at 5°C. The fish silage was fed to the sheep daily through a drench gun.

1.3 Animals, diet and treatments

In total 16 entire Coopworth Cross Romney male lambs (initial BW range of 39.5+/-1.4 kg; age 10 months) were selected from Lincoln University sheep unit. At selection, the lambs were weighed, ear tagged, and treated with nematode gastrointestinal anthelmintic oral drench, Matrix Hi-Mineral (Boehringer Ingelheim, Auckland), at recommended dose. They were then housed indoors in individual pens with individual water and feed source at the university animal house.

The 16 lambs were fed a basal diet of lucerne silage and water for 10 d to adapt them to the indoor feeding system. This was done in individual pens in the animal house on arrival. Then 14 d before entering the metabolism crates, their diets were changed to ryegrass, and group 1 began its 60 ml allocation of fish silage. Sheep were all fed to *ad libitum* intakes, with greater than 10% refusal daily maintained. The ryegrass used in this experiment was harvested daily from a paddock that was in its vegetative stage of growth. The 16 sheep were then put into the metabolism crates for 7 d of total urine and faecal collection. During Run 1, group 1 was fed a ryegrass diet at an allocation of 9 kg wet weight with a daily 60 ml drench of fish silage, while group 2 was fed a ryegrass diet at an allocation 9 kg wet weight. After Run 1, all sheep were returned to pens and the feed treatments swapped over, and the process repeated for Run 2.

1.4 Measurement and sampling

1.4.1 Feed

Each day that the lambs were in the metabolism crates during Run 1 and 2, the ryegrass refusals were weighed prior to allocation of fresh feed. These refusals were put into separate paper bags, labelled with the day and sheep number, then placed into an air fan oven at 65 °C to dry them to a constant weight, and used to calculate the daily feed intake of each lamb.

After cleaning out the refusals in the crate each day, fresh ryegrass was initially weighed and fed at 1000 h. On the bases of fresh weight of ryegrass refusals, the daily allocation of wet weight ryegrass allocation was moved to ensure at least 10% refusal by wet weight each day.

1.4.2 Urine and faeces

During both Runs 1 and 2 in the crates, total urine and faecal excretions were collected and weighed from each sheep, daily. Faecal and urine excretions were divided using a faecal separator, with collection containers beneath each separate crate. Each day 25% sulphuric acid was added to each urine collection container to acidify daily urine to a pH below 3.0. This was done to prevent NH_3 volatilization and microbial degradation of the urine metabolites.

Urine and faecal samples were collected daily before 1000 h. For urines, 5% was taken from each sample after the total volume was weighed. These were bulked and frozen each day at -20°C for further analysis. The faeces excreted by each sheep was weighed daily, and a 10% subsample was bulked and frozen each day at -20°C for further analysis. The remaining faeces were equally divided after mixing and put into duplicate sample bags for DM assessment.

1.5 Analysis

1.5.1 Feed

For both runs, the subsampled frozen ryegrass was freeze dried. They were then ground through a 1mm sieve screen centrifugal rotor mill (Retsch-ZM, Haan, Germany). There were then assessed for NDF, OM and N using wet chemistry methods.

1.5.2 Urine and faeces

At the conclusion of the trial, the urine samples were thawed and subsampled into 2 ml containers after thorough mixing. Faecal samples were thawed and dried at 65°C to a constant weight then ground through a 1mm sieve (Retsch-ZM, Haan, Germany) before further analysis. The NDF, OM and N content was obtained using wet chemistry methods, and the urinary purine values obtained using HPLC method of Chen et al. (1987).

1.6 Calculations

Feed, urine and faeces

While the sheep were in the metabolism crates, the daily refusal of their ryegrass were subtracted from their daily intake to establish a daily DM intake. The NDF, ADF, and CP intakes were then calculated from this value meaned across 7 d. Similarly, faecal excretion of NDF, OM and N was calculated and used to obtain digestibility and N balance values. The microbial protein and N outputs were calculated from estimates of the daily mean urinary purine outputs using the equations of Chen et al. (1987).

1.7 Statistical analysis

Statistical analysis for this data was carried out using GenStat (VSN Ltd, London). An analysis of variance (ANOVA) was carried out for DMI, OMI, DMD%, DOMD%, NDF/ DMD%, microbial N (g/ d), microbial N/ OM intake using the treatment as the factor and animal as the block structure. Least significant different (LSD) test was carried out to compare the means with significant differenced form ANOVA.

Results

1.8 Feed analysis

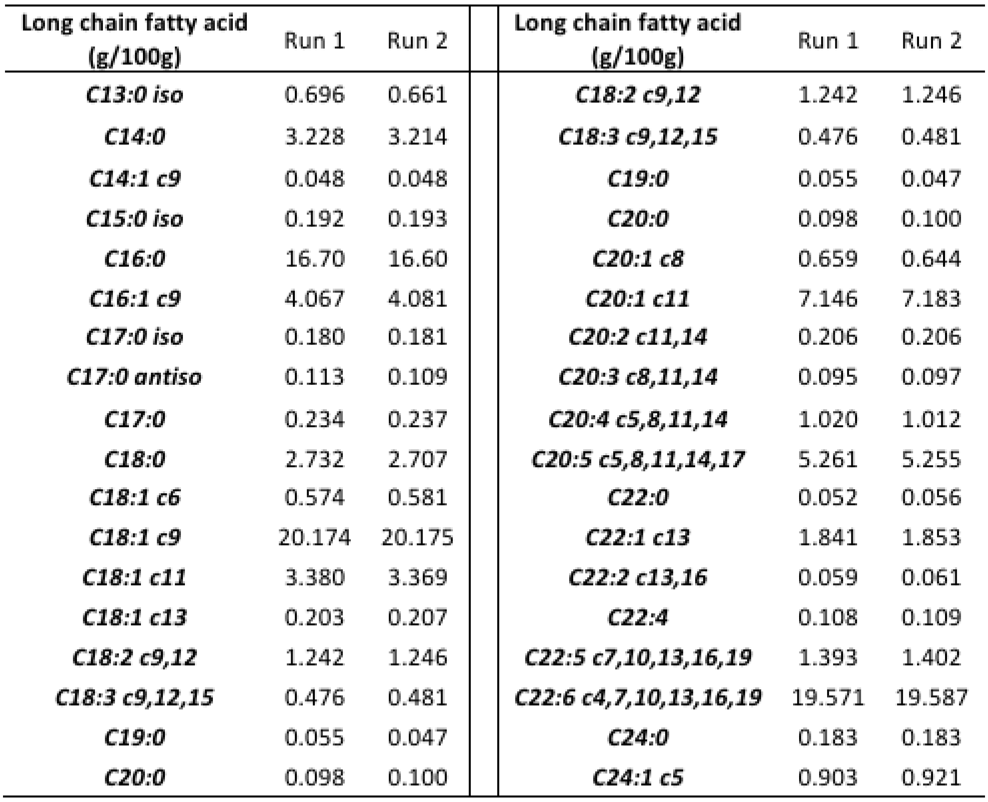
The results of the proximate analysis of the feed are displayed in Table 3. The fat [ether extract (EE)] content of the FS was above 20% and the NDF content of the grass was below 45%. The long chain fatty acids are displayed in Table 4; PUFA and in particular Omega 3 groups, were enriched.

Table 3: Feed composition

%	Run 1		Run 2	
	Pasture	Fish Silage	Pasture	Fish Silage
DM	16.4	28.1	20.1	32.5
OM	12.9	9.4	9.74	10.15
CP	11.75	63.31	8.56	63.25
NDF	42.1	0	42.3	0
EE	<4	>20	<4	>20

DM = dry matter; OM = organic matter; CP = crude protein; NDF= neutral detergent fibre, EE = ether extract

Table 4: Long chain fatty acids of fish silage



Long chain fatty acid (g/100g)	Run 1	Run 2	Long chain fatty acid (g/100g)	Run 1	Run 2
C13:0 iso	0.696	0.661	***C18:2 c9,12***	1.242	1.246
C14:0	3.228	3.214	***C18:3 c9,12,15***	0.476	0.481
C14:1 c9	0.048	0.048	***C19:0***	0.055	0.047
C15:0 iso	0.192	0.193	***C20:0***	0.098	0.100
C16:0	16.70	16.60	***C20:1 c8***	0.659	0.644
C16:1 c9	4.067	4.081	***C20:1 c11***	7.146	7.183
C17:0 iso	0.180	0.181	***C20:2 c11,14***	0.206	0.206
C17:0 antiso	0.113	0.109	***C20:3 c8,11,14***	0.095	0.097
C17:0	0.234	0.237	***C20:4 c5,8,11,14***	1.020	1.012
C18:0	2.732	2.707	***C20:5 c5,8,11,14,17***	5.261	5.255
C18:1 c6	0.574	0.581	***C22:0***	0.052	0.056
C18:1 c9	20.174	20.175	***C22:1 c13***	1.841	1.853
C18:1 c11	3.380	3.369	***C22:2 c13,16***	0.059	0.061
C18:1 c13	0.203	0.207	***C22:4***	0.108	0.109
C18:2 c9,12	1.242	1.246	***C22:5 c7,10,13,16,19***	1.393	1.402
C18:3 c9,12,15	0.476	0.481	***C22:6 c4,7,10,13,16,19***	19.571	19.587
C19:0	0.055	0.047	***C24:0***	0.183	0.183
C20:0	0.098	0.100	***C24:1 c5***	0.903	0.921

1.9 Dry matter intake

The DMI data of Run 1 and 2 are displayed in Table 5. There was no significant difference between treatment groups in either run, or both runs combined.

Table 5: Dry matter intake

DMI (g/DM)	Treatment 1	Treatment 2	*p-value*	LSD
Run 1	7701^{a} (226)	7415^{a} (140)	0.301	569
Run 2	7187^{a} (262)	7468^{a} (512)	0.633	1239
Mean	7444	7441	0.995	645

DMI = Dry matter intake; LSD = least standard difference. Between Run 1 and 2 significance was assumed at p>0.05, different superscripts denote significance. In parentheses is the standard error of the mean.

The OMI data of Run 1 and Run 2 are displayed in Table 6. There was no significant difference between treatment groups in either run, or both runs combined (P=0.721).

Table 6: Organic matter intake

OMI (g/DM)	Treatment 1	Treatment 2	*p-value*	LSD
Run 1	6725^{a} (203)	6527^{a} (132)	0.427	519
Run 2	6302^{a} (244)	6710^{a} (471)	0.454	1137
Mean	6514	6619	0.721	597

OMI = organic matter intake; LSD = least standard difference. Between Run 1 and 2 significance was assumed at p>0.05, different superscripts denote significance. In parentheses is the standard error of the mean.

1.10 Feed digestibility studies

The feed digestibility results for both treatment diets, in Run 1 and Run 2 are displayed in Table 7. The results show that there was no significant difference in either dry matter digestibility (DMD%) or digestible organic matter digestibility (DOMD%) in either treatment.

Table 7: Digestibility of treatment diets

	Treatment 1		Treatment 2		*p-value*		LSD	
	DMD%	DOMD%	DMD%	DOMD%	DMD%	DOMD%	DMD%	DOMD%
Run 1	86.4^{a} (0.6)	87.4^{a} (0.67)	84.5^{a} (0.9)	85.8^{a} (0.85)	0.101	0.156	0.024	2.35
Run 2	85.5^{a} (0.01)	86.0^{a} (1.51)	85.8^{a} (0.01)	86.5^{a} (1.10)	0.871	0.785	0.041	4.01
Mean	86.0	86.7	85.2	86.1	0.459	0.609	2.25	2.22

DMD = digestible dry matter; DOMD = digestible organic matter digestibly; SEM = Standard error of mean; LSD = least standard difference. Between Run 1 and 2 significance was assumed at p>0.05, different superscripts denote significance. In parentheses is the standard error of the mean.

The neutral detergent fibre DMD (NDF/ DMD%) of both treatment diets for Run 1 and Run 2 are displayed in Table 8 below. There was no significant difference in the NDF/DMD% for either treatment in both runs.

Table 8: Neutral detergent fibre digestibility of treatment diets in run 1 and 2

	Treatment 1	Treatment 2	*p-value*	LSD
Run 1	86.8^{b} (0.43)	85.5^{ab} (0.79)	0.159	1.908
Run 2	82.5^{a} (2.17)	82.6^{a} (1.58)	0.967	5.770
Mean	84.6	84.0	0.668	2.861

LSD = least standard difference. Between Run 1 and 2 significance was assumed at p>0.05, different superscripts denote significance. In parentheses is the standard error of the mean.

1.11 Nitrogen

Both the urine and faecal N excretion (g/ d) of both treatments for Run 1 and Run 2 are displayed in Table 9 below, and that of the ratio of urine N/ faecal N in Table 10. There was no significant difference in the urinal or faecal N excretion for either treatment in both runs.

Table 9: Urine and faecal nitrogen excretion

	Treatment 1		Treatment 2		*p-value*		LSD	
	Urine N (g)	Faecal N (g)	Urine N (g)	Faecal N (g)	Urine N (g)	Faecal N (g)	Urine N (g)	Faecal N (g)
Run 1	42.39	34.1	41.97	36.9	0.867	0.389	5.26	6.86
Run 2	23.75	28.6	29.21	28.3	0.05	0.926	5.45	7.98
Mean	33.07	31.4	35.59	32.6	0.164	0.616	5.116	7.11

G = grams; LSD = least standard difference; N = nitrogen. Between Run 1 and 2 significance was assumed at p>0.05.

Table 10: Ratio of urine nitrogen (g)/ faecal nitrogen (g)

	Treatment 1 (g)	Treatment 2 (g)	*p-value*	LSD
Run 1	1.266	1.161	0.289	0.204
Run 2	0.864	1.095	0.134	0.312
Mean	1.065	1.128	0.474	0.252

The total N excreted, and N efficiency, in both treatments for Run 1 and Run 2 are displayed in Tables 11-14 below. There was no significant difference in the total N excreted for either treatment in both runs, but N efficiency differed in Run 1 and the mean of runs.

Table 11: Total nitrogen excreted grams per day

	Treatment 1 (g)	Treatment 2 (g)	*p-value*	LSD
Run 1	76.5	78.9	0.634	10.69
Run 2	52.4	57.5	0.314	10.49
Mean	64.4	68.2	0.290	10.12

G = grams; LSD = least standard difference. Between run 1 and 2 significance was assumed at p>0.05.

Table 12: Total nitrogen excreted grams/ liveweight

	Treatment 1	Treatment 2	*p-value*	LSD
Run 1	2.15	2.154	0.974	0.287
Run 2	1.273	1.382	0.352	0.244
Mean	1.711	1.768	0.522	0.2546

Table 13: Total nitrogen excreted grams/ grams total weekly dry matter intake

	Treatment 1	Treatment 2	*p-value*	LSD
Run 1	0.00991	0.01068	0.210	0.00125
Run 2	0.00741	0.00792	0.586	0.001996
Mean	0.00886	0.00930	0.251	0.00159

Table 14: Nitrogen efficiency %

	Treatment 1 (%)	Treatment 2 (%)	*p-value*	LSD
Run 1	51.6	43.4	0.017	6.49
Run 2	52.9	42.4	0.117	13.54
Mean	52.3	42.9	0.012	10.14

The microbial protein production of both treatments in g/ d and g MCP/ OMI for Run 1 and Run 2 are displayed in Table 15 below. There was no significant difference in microbial protein production in g/d or g MPC/ OMI for either treatment in both runs.

Table 15: Microbial protein production in grams daily

	Treatment 1		**Treatment 2**		***p-value***		**LSD**	
	g/d	gMCP/OMI	g/d	gMCP/OMI	g/d	gMCP/OMI	g/d	gMCP/OMI
Run 1	16.43^{b} (1.45)	2.45^{b} (0.21)	17.53^{b} (0.51)	2.69^{b} (0.09)	0.489	0.308	3.320	0.490
Run 2	12.18^{a} (0.22)	1.96^{a} (0.12)	12.98^{a} (1.03)	1.97^{a} (0.15)	0.458	0.972	2.256	0.419
Mean	14.30	2.21	15.26	2.33	0.31	0.41	1.88	0.305

MCP = microbial crude protein; g = grams; kgDM = kilograms of dry matter; LSD = least standard difference. Between Run 1 and 2 significance was assumed at $p>0.05$, different superscripts denote significance. In parentheses is the standard error of the mean.

Discussion

1.12 Introduction

This was the first research to quantify the DMD, DOMD, NDF DMD and N use efficiency of ram lambs supplemented with fish silage (BioMarinus) at methane mitigation doses on a high intake, low DM pasture diet mimicking a traditional New Zealand dairy system diet. High quality, low DM ryegrass diets are known to have high rumen outflows (Saldias, 2014) which is a key consideration that needs to be addressed when choosing suitable methane mitigation.

Fish oil supplementation has the potential to be used widely throughout New Zealand's livestock production systems as a methane mitigation tool, however, oil supplementation has been reported to have adverse impacts on the rumen environment, and this supplement would increase the CP concentration in an already high CP diet. Therefore, it is important to quantify the effect that has on DMD, DOMD, NDF DMD and N excretion and efficiency. Previous experiments have demonstrated a negative effect of oil on fibre degradation in the rumen (Bhatt *et al*., 2011; Harventine & Allen, 2006; Jenkins & Palmquist, 1984), however, there are no previous published reports of N excretion or N efficiency in an New Zealand system.

The results of this experiment demonstrated no negative effects of BioMarinus fish silage supplementation on intake, DMD, DOMD, DMD NDF or N excretion of ran lambs despite having a high (>20%) oil content and a high (>40%) CP content. This research demonstrated that there is no apparent disincentive in digestibility or N excretion observed when supplementing ram lambs with BioMarinus fish silage at a dose suitable for enteric methane reduction.

1.13 Intake

The DMI and OMI were not reduced by fish silage supplementation in this experiment (Table 5 and 6). Previous studies have demonstrated that fish oil supplementation can decrease overall DMI in ruminants (Wachira *et al*., 2002; Fieves *et al.,* 2003; Toral *et al*., 2010a). It has been speculated that ruminant dietary fat supplements can reduce NDF digestibility in the rumen as lipid particles create direct competition with rumen microbes for absorption onto feed particles, or through direct adverse impacts on cellulolytic rumen micro-flora (Benepal, 2012). Reduced NDF DMD results in increased rumen fill, which in turn reduces DMI (Beauchemin *et al*., 2020).

Increasing fish oils in the diet have also been attributed to increases in particular biohydrogenation intermediaries that are potentially toxic to rumen microbiota, also reducing rumen fermentation

(Toral *et al.,* 2010a). This would be a significant disadvantage to feeding fish silage as it would decrease overall animal production which is key for farm profitability.

Toral et al., (2010a) who recorded reductions in DMI when supplementing ruminants with fish oil was feeding fish oil at a rate of 10 g/kg fresh weight of their diet. This dose rate equated to 1.7 g of oil / kg metabolic weight. The dose rate for this experiment was 60 ml fish silage (~20% oil and ~60% CP), representing 0.65 g oil /kg metabolic BW. This is the recommended dose rate needed to achieve effective methane mitigation with a forage diet (Gibbs, 2010). Toral *et al*., (2010a) fed over double the recommended dose rate of oil for methane reduction, and therefore recorded a reduction in DMI. This evidence demonstrates that the dose used in this experiment was no substantial enough to reduce DMI.

Mertens (1996) proposed that due to the slow degradation rate of NDF, daily DMI would be restricted by NDF intake once a threshold of approximately 1.2% of body weight was reached in forage based diets. The NDF intake for Treatments 1 and 2 averaged 1.20% and 1.16% respectively. Therefore, it is likely that NDF was the limiting factors for DMI, rather than the adverse impacts from oil supplementation as seen in other research.

The results of this study compare to similar results that were found by Marsetyo *et al*,. (2012) who investigated the effect of copra and rice bran supplementation on BW gain and feed intake of weaned cattle. Rice bran and copra supplements are known for their high fat contents with rice bran potentially containing between 15–24% fat (Stein *et al*., 2015) and copra containing around 71% crude fat on a dry weight basis (Ghosh *et al*., 2014). In this study, fat was fed at a rate higher than 5% BW. The results of this particular experiment demonstrated that the supplementation of fat at a rate higher than 5% did not result in a decrease in DM intake, in fact, DMI was increased compared with the control diet of pasture alone.

In this situation, fish silage was used as a methane mitigation supplement, rather than to increase tissue fatty acid composition as in the experiment of Wachira, *et al*., (2002), or to increase energy supply using PUFA as in the experiments of Pereira *et al*., (2022) who both saw reductions in DMI. The results presented in this study demonstrated that the rate of fish silage required for effective methane mitigation did not reduce DMI or OMI.

1.14 Feed digestibility

This is the first experiment to measure the digestibility of feed components of ram lambs fed a ryegrass diet, supplemented with fish silage to be used as a methane mitigant. Supplementation of fish silage did not impact DMD, DOMD, or NDF DMD (Table 7 and 8). This result is in contrast with the results of various studies where a reduction in NDF digestibility was observed when ruminants were fed fat supplements (Bhatt *et al*., 2011; Harventine & Allen, 2006; Jenkins & Palmquist, 1984).

Reductions in the digestibility of NDF would likely have negatively impacted on animal production, as NDF made up 41.1% of the feed. Therefore, a reduction in NDF digestibility would strongly reduce the availability of energy for the lambs. This would also relate to dairy systems, due to dairy pasture typically containing approximately 40% NDF (De Veth & Kolver, 2001, Saldias, 2014). In this experiment, fresh ryegrass was harvested daily and fed at *ad libitum* to mimic a traditional New Zealand dairy diet as closely as possible.

There are various mechanisms that cause dietary fat supplementation to depress rumen digestibility. Macleod (1981) stated that when fat intake exceeds 5% of the diet, there was reduced activity of cellulolytic organism in the rumen which are essential for the digestion of cellulose in the rumen. This has been contested by additional research who did not demonstrate this, despite using higher fat concentrations in the diet (Doreau & Chilliard, 1997). It has also been contested by more recent work that did not demonstrate adverse impacts on NDF DMD in diets high in PUFA concentrations when ruminants were fed C4 pasture diets (Costa *et al*., 2017)

Previous research has not investigated the effect of PUFA on NDF digestibility in low DM ryegrass diets with large rumen volumes and a fast rumen passage rate. High rumen passage rates result in a rapid turnover of oils (Benepal, 2012). There is some evidence that high rumen passage rates dilute PUFA more than they might do in TMR diets.

It is likely that in this experiment, the rate of fish silage supplementation was insufficient to elucidate the reductions in DMD, DOMD or NDFDMD observed in the other research. Typical South Island pasture crude fat concentrations are 4-6% DM (Saldias, 2014). The total additional fat added in this experiment was 0.65g metabolic BW. This is likely to have consistently maintained fat below the concentrations used in the experiment of Macleod (1981), which is likely to be the reason for the difference in digestibility observed.

These results demonstrate that there is no disincentive to supplementing ruminants with 60 ml of BioMarinus fish silage in order to reduce methane production. There appears to be no negative effects on rumen efficiency and therefore animal production. The results obtained from this experiment show that expectations that fish oils could negatively affect NDF digestibility in pasture based systems may be over-stated.

1.15 Nitrogen

This is the first experiment to measure the urinary and faecal N losses of ram lambs fed a ryegrass diet, supplemented with fish silage to be used as a methane mitigant. Despite the high CP concentration of fish silage there was no increase in urinary or faecal N losses (Tables 9-12).

New Zealand dairy pasture diets generally contain excess dietary N, which is further exacerbated by the application of N fertilisers to boost pasture production (Pacheco & Waghorn, 2008). Excess dietary N results in negative environmental impacts as it leaves the body through the urine which can then be leached through the soil into water ways or volatilised into nitrous oxide and lost to the atmosphere. It is therefore a positive result environmentally that the increase of CP through the supplementation of fish silage did not increase urinary or faecal losses.

The high CP concentration of the pasture means that excess CP is degraded to ammonia and excreted via urine. Additional protein in the diet would be expected to increase this N excretion through the urine. The lack of increase in urinary N excretion suggests that the additional N from the fish silage was not degraded in the rumen, and was instead, rumen bypass protein.

This is supported by Ferndandez *et al*., (2004) who supplemented growing goats with 60 g/ kg DM of fish oil fat which was rich in rumen protected polyunsaturated fatty acids with a CP content of 208 g/ kg DM. Fernandez *et al.* (2004) found that urinary N losses were lower in the fish oil diet compared to the control diet, concluding that fish silage is degraded in the small intestine rather than the rumen. Greater N retentions were also observed in the fish oil diet compared to the control diet both in relation to the quantity of N ingested and the quantity absorbed because the energy value of the fish silage supplement may also increase BW gains.

The lack of increase in faecal N excretion also suggests that the protein from the fish silage was absorbed in the small intestine. This results in an increased metabolizable protein supply to the animal which in turn, can increase animal production. Crawford *et al*., (2020) included a corn-fish meal supplement to grazing lambs containing protein in the form of RUP which resulted in increased bodyweight gains when supplement was increased from 1 to 2% BW, demonstrating increasing animal production with increased RUP present in the diet.

Despite feeding a higher CP supplement, the results of this experiment saw an increase in N use efficiency (Table 13). Orskov *et* al., (1987) reported that CP in fish silage was in the form of RUP rather than RDP. As a result of this, fish proteins are poorly degradable in the rumen, and leave the rumen in the same form they entered in, to later be broken down and absorbed in the small intestine. Therefore, the additional CP entering the system was not degraded and lost as ammonia from rumen microbial degradation, rather it was likely directly absorbed as amino acids from the small intestine. An increased N load in the diet appeared to result in increased N use efficiency as N excretion did not increase despite increased dietary N intake. This was supported by the lack of difference in microbial protein production between treatments (Table 15).

This is a positive result, because if other lipids were used as methane mitigants (such as sunflower oil or linseed oil) (Vargas *et al*., 2011; Jouany *et al.,* 2008) through the same mechanism as fish oils, the increased CP load to the diet will not be in the form of RUP, so can therefore decrease the animals N use efficiency, and increase the N excreted in both the urine and faeces. Increasing CP supply through fish silage and in turn receiving increased metabolizable protein supplied with no increased N excreted is a positive result.

1.16 Experimental design and limitations

In this experiment, a cross over experimental design was used to ensure the statistical power of the experiment was maximised, within practical constraints such as number of sheep to be used practicably. During this experiment, seasonality posed a challenge in terms of maintaining consistent pasture quality. Between Run 1 and Run 2, DM varied 3.7% with Run 1 having an average pasture DM of 16.4% and Run 2 having an average pasture DM of 20.1% (Table 3). This variation in pasture DM is likely to have influenced rumen passage rate and may have influenced the continuity of results.

Due to the nature of this experimental design, requiring animals to be on a fresh ryegrass diet for over 28 days makes it challenging to maintain consistent DM. However, the NDF of the fresh ryegrass varied only 0.2% between runs (42.1% in Run 1 and 42.3% in Run 2) which demonstrates adequate continuity of NDF concentration between runs. This allows us to make more confident comparative conclusions between runs and treatments in terms of NDF digestibility effects.

When using a cross over experimental design, it is important to ensure the number of animals used is adequate to detect small changes between treatments. Based on a repeated measures cross-over design including a sequence (Run 1,2) for carry-over effect a power analysis with an effect size Cohen's D ƒ=0.5, β/α ratio = 1, total sample size n = 16, number of groups = 2, number of measurements = 4, correlation among repeated measures = 0.5, gives a statistical power (1-β) of 0.845 or 84.5%. This shows that 8 sheep per group was adequate to represent and pick up small changes between treatments.

Ram lambs were used in this experiment in order to best represent NZ dairy intakes. The intent was to avoid using stable BW wethers as relative intakes would have been lower due to the lack of growth. Furthermore, there lambs were in a weight bracket which allowed adequate CP supply from pasture alone, which would not have been true for smaller ram lambs. The ram lambs averaged a DMI of ~3% BW in Run 1 and ~2.5% BW in Run 2. Mimicking these high intakes helped to get a more accurate representation of the effect that fish silage would have in a high intake, high rumen flow rate dairy diet that is common in New Zealand dairy systems (Saldias, 2014). Cattle were not used in this experiment because of the logistical difficulties of feeding 16 cattle in crates, along with the cost of obtaining enough fresh feed for them. Therefore these lambs were the best, most practicable option to represent a high intake diet, similar to NZ dairy systems.

This experiment being undertaken in crates had the potential to cause some disturbance to the lambs usual grazing behaviour as this was a different environment and feeding program to what they are used to. Despite being in crates, the high DMI observed in both runs demonstrates the lambs were still maintaining high intakes in this environment.

1.17 Future research

The main cause of increased methane production is generally a result of fibre degradation. This experiment was undertaken using a comparatively high NDF diet, with sheep eating to their maximum NDF intakes of ~1.2% on a ryegrass diet meaning they were on a maximum methane production diet. In a TMR diet, ruminants would only eat around 1%. Subsequently, for future research, what increased dose of fish oil would be required to cause a reduction in in NDF DMD in this *ad libitum* ryegrass diets should be investigated.

As this experiment was only undertaken on lambs, it would be beneficial to repeat this trial on cattle to quantify the effects that a fish oil dose supplemented to the amount needed to reduce cattle methane on their intake, DMD, DOMD, DMD NDF and N efficiency. Replicating this study on cattle would be logistically challenging in terms of keeping cattle in metabolism crates to measure intake and excretion accurately. Replicating this trial with cattle may have to be undertaken in grazing conditions to make it more logistically viable.

This experiment was a short term study, only measuring the effect of fish silage over less than a month. For the benefit of understanding the long term effects, specifically on the effect on animal production, a long term study should be undertaken to quantify this.

1.18 Summary and Conclusions

The primary findings of this experiment were that a 60 ml drench of BioMarinus fish silage had no effect on rumen DOMD, DMD or NDF digestibility, therefore having no observed negative effect on rumen function and no apparent cost to animal production. Although fish silage has a high N content, increasing total nitrogen intake, there was no increase on urine or faecal N excretion. The N efficiency increased when fish silage was fed.

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