Methane mitigating feed additives in future dairy production

Consumer and producer attitudes and potential effects on dairy products

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Master's Thesis • 30 credits
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Agricultural Programme - Food Science
Molecular Sciences, 2022:54
Uppsala, 2022

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Metan inhiberande fodertillskott i framtida mejeriproduktion – konsument och producent attityder till fodertillskotten och potentiell effekt på mejeriprodukter

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Credits:	30 Credits
Level:	Second cycle, A2E
Course title:	Master thesis in Food science – Agricultural program - Food
Course code:	EX0877
Programme/education:	Agricultural Programme - Food Science
Course coordinating dept:	Department of Molecular Sciences
Place of publication:	Uppsala
Year of publication:	2022
Copyright:	All featured images are used with permission from the copyright owner.
Title of series:	Molecular Sciences
Part number:	2022:54

Keywords:	Enteric methane, mitigating strategies, rumen metabolism, 3-Nitrooxypropanol, *Asparagopsis taxiformis*

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Abstract

Enteric methane emitted from ruminant metabolism is the most prominent greenhouse gas produced in livestock production. Mitigation strategies in the form of different types of methane reducing feed additives are approaching the market in near future. The effects of implementing the feed additives into the dairy sector was evaluated in this study. The chemical synthesized compound 3-Nitrooxypropanol and the algae *Asparagopsis taxiformis* are the most prominent methane reducing feed additives with the potential of reducing enteric methane production up to 30% without reducing milk yield or nutrient utilization. Effects on volatile fatty acids, protein profile, and fatty acid composition observed encourage further studies on milk quality to ensure that dairy products are not affected. The concentration of different fatty acids and protein shifted, while the total concentration of fat and protein in the milk was unaffected. Compounds deriving from methane mitigating additives in the form of bromoform and nitrites have known health concerns in larger quantities. Accumulation and excretion of these compounds is an area of research needed to ensure a safe long-term usage. The possibility of economic foundation, and survey results showing optimism amongst both consumers and producers support further studies for implementation of methane reducing feed additives into the dairy production.

Keywords: Dairy cows, enteric methane, mitigating strategies, rumen metabolism, 3-nitrooxypropanol, *Asparagopsis taxiformis*

Sammanfattning

Metan som bildas och släpps ut från idisslarens metabolism är den mest framträdande växthusgasen som produceras i nötkreaturproduktion. En ny strategi för att minska metanutsläpp är i form av metanreducerande fodertillsatser som i olika form närmar sig marknaden inom en snar framtid. Effekterna av att införa fodertillsatserna i mejerisektorn utvärderades i denna studie. Den kemiskt syntetiserade föreningen 3-Nitrooxipropanol och algen *Asparagopsis taxiformis* är de mest framträdande metanreducerande fodertillskotten med potential att minska metanproduktion med upp till 30 % utan att minska mjölkutbytet eller näringsutnyttjandet. Effekter på flyktiga fettsyror, proteinprofil och fettsyrasammansättning har observerats och uppmuntrar ytterligare studier på mjölkkvalitet för att säkerställa att mejeriproduktionen inte påverkas. Koncentrationen av olika fettsyror och protein skiftade, medan den totala koncentrationen av fett och protein i mjölken var opåverkad. Föreningar som härrör från metanreducerande tillsatser i form av bromoform och nitriter har kända hälsoproblem i större mängder. Ackumulering och utsöndring av dessa föreningar är ett fortsatt forskningsområde som behövs för att säkerställa en säker långtidsanvändning. Möjligheten till ekonomiskt stöd för dessa tillskott samt enkätresultat som visar optimism bland både konsumenter och producenter stödjer ytterligare studier för införande av metanreducerande tillsatser i mejeriproduktionen.

Nyckelord: Mjölkkor, metan, reducerande strategier, fodermetabolism, 3-nitrooxipropanol, *Asparagopsis taxiformis*

Table of contents

List of tables

List of figures

Abbreviations

3-NOP	3-Nitrooxypropanol
AA	Amino acids
ACC	Acetyl-CoA carboxylase
ADP	Adenosine triphosphate
CCP	Colloidal calcium phosphate
CE-MS	Capillary electrophoresis–mass spectrometry
CO_2	Carbon dioxide
CH_4	Methane
CLA	Conjugated linoleic acid
CP	Crude protein
DM	Dry matter
DMI	Dry matter intake
ER	Endoplasmic reticulum
FTIR	Fourier transform infrared
GHG	Greenhouse gas
H_2	Dihydrogen
LAB	Lactic acid bacteria
MCR	Methyl coenzyme M-reductase
MFG	Milk fat globules
MO	Microorganisms
N	Nitrogen
NH3	Ammonia
OM	Organic matter
PTMs	Post-translational modifications
RDP	Rumen degradable form
RUP	Rumen undegradable form
SCFA	Short-chain fatty acids
TAG	Triglycerides
VFA	Volatile fatty acids

1. Introduction

By 2050, the expected global demand for milk will increase by 58% and meat as much as 78% compared to the demand in 2010. The numbers reflect the growing world population, increasing urbanization, and diet becoming more versatile in the growing middle class (Gerber 2013). The global impact of the livestock supply chain is estimated to account for about 14.5% of the total greenhouse gas (GHG) emissions caused by humans (Spaull & Napolitano 2016). Where enteric methane (CH_4) accounts for around 39% of the total GHG emissions in the livestock sector (Gerber 2013). An increased production enforces development of a more sustainable livestock production.

Swedish agriculture generated approximately 6.9 million tonnes of carbon dioxide equivalents emitted in 2020. Where CH_4 emissions from enteric digestion accounted for 42% of the sector's total emissions (Naturvårdsverket 2022c). Enteric CH_4 are formed from residual products in the fermentative metabolism of feed in the rumen. A group of Archaea called methanogens in the rumen have methane as their metabolism end product, functioning as a hydrogen sink and hindering accumulation of hydrogen in the rumen (Agarwal et al. 2015).

Reducing GHG emissions is a global responsibility. Hence, there are global climate targets such as the Paris Agreement to mitigate global GHG emissions limiting global warming to 1.5℃, and a maximum of 2℃. Strategies to reach the set aim are conducted on various levels: EU level (European Green Deal) (European Commission 2021), national level (climate strategic policy) (Naturvårdsverket 2022a), or private sector (Science-based target initiative) (WRI 2022). All based on strategic plans on how to reach the aim of the Paris agreement.

Strategies to mitigate emissions from livestock production could be increasing production efficiency, improved manure management and feed production (Gerber et al. 2013). A new approach is to mitigate livestock production of enteric methane by altering the ruminant metabolic processes with feed additives. Different types of additives approaching the market have proven mitigation effects on methane production with variations in effects. This study will cover the function and effect of two different groups of additives: chemically produced additives (3-NOP, dicarboxylic acids, nitrates), and plant derived additives (algae, plant secondary compounds) (Beauchemin et al. 2020). In an attempt to give an overview of those additives that may be relevant for use in dairy farming in the near future.

1.1 Aim of the study

The aim is to evaluate potential effects on usage of the most prominent methane reducing feed additives in dairy production. Could additives mitigating enteric methane alter the composition and properties of milk, and thus affect dairy processing? What are the opportunities and challenges that need to be addressed in the issue?

2. Background

The objective of the background is to give a foundation of the ruminant's digestive system and feed digestion resulting in the precursors utilized for milk synthesis and enteric methane production. The background concludes with the environmental impact of milk production.

2.1 Dairy cows feed

Dairy cows need a versatile and well-balanced diet of high quality with the right structure to produce food and maintain health. The energy and nutrients in the feed are found in the feed's dry matter (DM), and the constituents that the ruminants can utilize are the dry matter intake (DMI) (Kennedy et al. 2009). The feed given to dairy cows can be divided into three categories: compound feed (concentrates), feed additives, and feed raw materials (forages) (Jordbruksverket 2022).

2.1.1 Forages

Good quality ley provides dairy cows with a significant part of their daily nutritional needs and are fed around 50% of the ratio of high producing dairy cows (Granström et al. 2022). The ley contains mixtures of grass and legumes, providing a rich source of carbohydrates, fibres, energy, and protein (Granström et al. 2022), providing non-synthesizable unsaturated fatty acids, linoleic acid (C18: 2), linolenic acid (C18: 3), and conjugated linoleic acid (CLA), with largest quantities in early cuts. Seasonal variations is seen in ratio of unsaturated and saturated fatty acids (Lindmark Månsson et al. 2006), with increasing amount of unsaturated fatty acids during the summer months with a feed ratio high in fresh forages. Common mixture of ley in Sweden is the mix of timothy grass and red clover (Spörndly et al. 2016). Legumes have a high decomposition rate leaving the rumen at a higher rate enabling a higher feed intake leading to increased milk yield (Johansson & Arnesson 2018).

Early cuts of pastures have a high nutrient bioavailability of crude protein (CP) and digestible fibres (Jordbruksverket 2014). Forages produced in Sweden are preserved predominantly as silage (Spörndly et al. 2016), and to a lesser extent as hay (Lärn-Nilsson & Malm 2022). Silage at 30-60% DM is packed in anaerobic storage in pits, bales or tubes enabling lactic acid bacteria (LAB) to ferment soluble carbohydrates such as glucose, sucrose, and fructose to form lactic acid, lowering the pH value. Rapid ensilage is beneficial in preventing unwanted protein degradation (Johansson & Arnesson 2018). Silage additives can be added at harvest to ensure optimal ensiling. The lower pH secures the fodder from growth of unwanted bacteria and yeasts. Both timothy grass and red clover have a high sugar content, advantageous in the ensiling process (Spörndly et al. 2016).

2.1.2 Concentrates

Concentrates are generally fed as a complement to forages to balance the nutritional requirements of the ruminants and is fed based on a feed evaluation (Lärn-Nilsson & Malm 2022), consisting of mainly cereals, legumes, and supplements of vitamins and minerals (Lärn-Nilsson & Malm 2022). Concentrates increases the protein and energy content of the feed (Jordbruksverket 2022). To some extent, by-products are fed as concentrates such as rapeseed meal or distiller's grain from other productions (Karlsson 2020). Oil crops fed as concentrates, such as rapeseeds can be given up to 5% of the total feed ration, providing enough fat content as energy for the microbes in the rumen (Johansson & Arnesson 2018). Legumes contain various amount of complex protein binding agents such as tannins, forming rumen stable proteins, delaying protein absorption to the fourth stomach (Johansson & Arnesson 2018) Feeding a high ratio of concentrate with ruminal-undegradable feed protein could supply the cow with up to 50% of its uptake of amino acids (AA). Though the majority of AA uptake are microbial proteins formed by the microorganisms in the rumen (Schwab & Whitehouse 2022). Adding energy in large quantities in the form of concentrates in milk production can, however, over time contribute to a surplus in the plant nutrient balance (Johansson & Arnesson 2018).

2.1.3 Feed supplements and additives

Feed supplements and additives could be used to improve the quality of feed for the dairy cows further (European Commission 2022b). The feed ratio can be complemented with feed supplements or additives approved for their use by the EU. A register states how the supplement or additive may be used and to what extent (Jordbruksverket 2022). Supplements in the EU include nutrients such as vitamins, minerals, and AA, added to ensure an adequate nutritional intake and physiological functions. But they are not used to alter physiological properties (EFSA 2022). Additives fed to ruminants can alter and improve physiological properties, improving animal production, performance, well-being, or environmental impact. The additives are categorized into different groups based on properties. Methane reducing feed additives are categorized as zootechnical additives as they are used to achieve a positive environmental impact by improving or altering the performance of healthy animals (European Parliament 2021).

2.2 Feed metabolism in ruminants

2.2.1 Rumen fermentation

The feed is chewed and mechanically broken down and mixed with the saliva and swallowed through the oesophagus to the anaerobic rumen reticulum. The rumen microorganisms (MO) begin fermentative metabolism of the feed. Muscle contractions in the stomach, mix the feed with the gastric fluid. The contraction triggers the cows' rumination, where part of the feed is pushed back up into the mouth to be chewed and then swallowed again. The rumination enables increased nutrients extraction from the feed being processed several times. Feed hard to digest as hay, undergo longer rumination compared to more easily digestible feeds such as fresh forages (Hobson & Stewart 1988). The rumen MO´s degrade and metabolize the feed into smaller constituents providing nutrients for uptake through the small intestine or directly through the walls of the rumen. The complex ecosystem of the rumen microbiota is highly diversified and have developed a symbiotic coexistence through metabolic processes with their host, providing the bovine with nutrients essential for the animal's health (Morgavi et al. 2010). The microorganisms consist of a variety of strains of bacteria, fungi, methanogenic archaea, protozoa, and bacteriophages (Janssen 2010; Morgavi et al. 2010). The inside of the rumen wall forms a large surface primarily permeable for uptake of volatile fatty acids, water, and part of the nutrients derived from the fermentation. The microbes use some of the nourishment of the feed for their own use to grow and multiply. The processed feed and part of the microbes are flushed to the acidic abomasum where nutrients such as microbial protein are released for uptake. The feed then proceeds to the small intestine where further breakdown and uptake of nutrients takes place (Hobson & Stewart 1988).

2.2.2 Metabolism of the different constituents of the digested feed

Protein metabolism

The protein content in the feed is often described by crude protein (CP) content which is estimated from the total amount of nitrogen compounds in the feed. The protein's synthesis requires a substantial amount of energy and CP. The protein can be in rumen degradable form (RDP) or rumen undegradable form (RUP). Around two thirds of the RDP (Stelwagen 2011) is metabolized by proteolytic microorganisms in the form of bacteria, protozoa, and anaerobic fungi. The RDP is broken down by enzymes hydrolysing protein peptide bonds, degrading protein into peptides, AA, and ammonia (Pfeffer & Hristov 2005; Johansson & Arnesson 2018). The protease activity is diverse and works in a synergistic manner in the rumen.

The main bacterial proteases present could be accompanied by other plant proteases consumed from the diet that could affect the rumen proteolytic activity (Pfeffer & Hristov 2005). The hydrolysed nitrogen compounds are utilized directly in the rumen, synthesized by the microorganisms into microbial proteins appropriate for utilization by the ruminant. The microbial proteins, together with one third of RDP escaping degradation in the rumen (Stelwagen 2011), flow to the small intestine for further hydrolysis and absorption (Janssen 2010). The microbial proteins account for around 50% of the total AA uptake into the bloodstream (Moss et al. 2000). The synthesis and protease activity requires energy from carbohydrate metabolism, used by microbes in the formation of new microbial proteins (Johansson & Arnesson 2018). The formation of microbial proteins ensures that the cows receive their essential proteins, even from feed with poorer protein quality (Stelwagen 2011). Essential AA in feed protein, milk protein and microbial protein are Arg, His, Ile Leu, Lys, Cys, Met, Phe, Thr, Trp, and Val. Limiting AA in milk protein secretion are Lysine and Methionine (Schwab et al. 1976). Increasing the amount of CP in the feed intake can increase the production of milk and protein amounts to a certain limit (Colmenero & Broderick 2006).

Some of the hydrolysed AA are not processed by the MO, leaving them to be deaminated forming a nitrogen (N) loss in the form of ammonia (NH3). The excess nitrogen diffuses out of the lumen (rumen wall), passing the liver and ends up as urea in excreta (Colmenero & Broderick 2006). A feed with excess N in relation to energy increases the release of ammonia into the environment (Pfeffer & Hristov 2005). A marker for overfeeding protein is milk urea, with extra cost for the producer as a result.

Fat metabolism

The degradation of fat molecules in the feed begins by the weak activity of lipase in the saliva when the feed is mechanically chewed. The fat molecules are hydrolysed to butyric acid among other short-chain fatty acids (SCFA). Vitamin B, C and K are also formed in the rumen. The carotene in feed goes directly from the rumen to the intestine epithelial cells to form vitamin A (Agarwal et al. 2015). The hydrolyzation of fatty acids continues in the rumen, with glycerol used as energy for the microbes releasing the fatty acid. Unsaturated fat is dominant in the feed of the ruminants in both grains and forages and is toxic to most microbes in the rumen (Agarwal et al. 2015). Through biohydrogenation in the rumen by the addition of hydrogen ions to the unsaturated fat, forming saturated fats mainly palmitic (16:0) and stearic acid (C18:0) detoxify the unsaturated fat (Agarwal et al. 2015). Excess fat in the feed ratio reduces the function of the rumen and the fat cannot undergo biohydrogenation to a sufficient extent. The result is a reduced dry matter intake (DMI), reduced fibre degradation and reduced ruminant performance (Agarwal et al. 2015).

Carbohydrate metabolism

Carbohydrates are the main energy source of the ruminant. It is divided into non-structural carbohydrates such as starch and sugar, and structural carbohydrates such as hemicellulose and cellulose. The MO in the rumen ferment and degrade the structural bonds of the carbohydrates to simple sugars. The simple sugar molecules are used as energy for the microbes and allow the microbes in the rumen to grow and increase in number (Pfeffer & Hristov 2005). The breakdown of carbohydrates results in the synthesis of energy-rich molecules such as adenosine triphosphate (ATP). The ATP are used in further fermentation processes in the rumen. The fermentation of feed constituents produce residual products in the form of volatile fatty acids (acetic, propionic, and butyric acids), dihydrogen (H_2), methane (CH_4) and carbon dioxide (CO_2) (Hobson & Stewart 1988; Agarwal et al. 2015). A deficiency in energy leads to a decrease of NH_3 uptake from the rumen and reduction of microorganisms, and ultimately increase in NH_3 lost in urine and excreta (Pfeffer & Hristov 2005).

Volatile fatty acids synthesis

Volatile fatty acids (VFA) produced in the fermentative metabolism give the ruminants energy and affect the milk's fat and protein synthesis. Acetic and butyric acids are precursors in the synthesis of fatty acids (≤C16), and propionic acid is precursor in the gluconeogenesis in ruminants. Different carbohydrates result in different proportions of the VFA acetate, propionate, and butyrate (Janssen 2010).

The metabolism of starch mainly forms propionate. Concentrates and maize silage are starch rich feeds and generally the ratio of propionate increases in the rumen with the amount of concentrates in fed. Forages contain large amount of structural carbohydrates such as hemicellulose and cellulose. When broken down in the rumen a large proportion of acetate is formed. The acetate is absorbed from the rumen wall directly to the liver where the acetate is utilized as a building block for fat synthesis in either mammary gland to milk fat or for storage in the adipose tissue. Reducing the ratio of acetic acid by increasing concentrate in feed affects the amount of milk fat produced by changing the ratio of VFA. The butyrate ratio remains stable independent of forage and concentrate ratio and is used in the fat synthesis where one C2 at a time builds up butyrate (C4) to palmitic acid (C16) (Agarwal et al. 2015).

A feed high in forage affects the rumen ratio of bacteria, by containing high numbers of bacteria such as fibrolytic microbes, which degrade abundant structural carbohydrates. The fibres are broken down at a slower rate than starch degraded by lactic acid bacteria, but more energy molecules can be extracted from those slowly degraded structural carbohydrates. The amount of lactic acid bacteria that metabolise starch into simple soluble sugars is found to a lesser extent in the rumen.

This is due to the relatively lower starch content in forages compared to structural carbohydrates (Hobson & Stewart 1988).

2.2.3 Milk synthesis

After the feed has been metabolized in the digestive tract, important nutrients have been extracted to the bloodstream carrying precursors to produce milk. The nutrients have either diffused from the rumen to the bloodstream and liver for further synthesis or have been taken up from the small intestine directly to the bloodstream. The variations in concentration of constituents in cow's milk are low, but its composition can be partly influenced by genetic factors, stage of lactation, or disease. The variations can be seen in differences in the constituents of the milk. An external factor such as feed mainly affects milk yield, and the fat content and composition (Walstra et al. 2005).

Milk components and precursors

Lactose

Propionates carried to the liver are through gluconeogenesis formed into glucose (Blowey & Edmondson 2010). Glucose is transported to the udder and epithelial cells where a part of the glucose is converted to galactose. Glucose and galactose are used to synthesize lactose in the Golgi apparatus. Lactose is transported through the cytosol in vesicles and released into the alveolar lumen. Lactose functions as the main osmotic determinant of milk by regulating uptake of water by osmoregulatory property. The vesicles become hypertonic from the lactose, drawing water into the vesicles from the cytosol creating an equilibrium. The more lactose produced the more water is drawn to the vesicles and are released into the alveolar lumen. Thus, the osmoregulatory properties of lactose regulate the volume of milk produced (Walstra et al. 2005; Stelwagen 2022).

The feed's impact on lactose production thus affects the volume of milk produced rather than the content of lactose in the milk. Glucose reaching the mammary gland can be affected by the ratio of forages and concentrates fed and the milking frequency. Reduced glucose delivered to the mammary gland results in lower lactose yield, and reduced volume of milk produced (Stelwagen 2022).

Lactose function in milk properties and dairy processes

Lactose gives the sweetness to the milk and can affect the sensory properties of fermented products. Lactose can be extracted from milk products for those who are lactose intolerant (Walstra et al. 2005). In cheese production the lactose functions as an energy source for bacteria strains such as LAB required in the fermentation processes, where approximately 20% of the total lactose content is used by the bacteria. A large part of the remaining lactose is dissolved via synereses in the whey solution (Simpson et al. 2012).

Milk protein

The largest proportion of protein in milk is synthesized in the mammary glands. The synthesized proteins are grouped into caseins (α-casein, β-casein, κ-casein, and γ-casein) and whey proteins (mainly α-lactalbumin and β-lactoglobulin). The absorbed AA are synthesized into encoded proteins in the endoplasmic reticulum (ER) due to induced gene expression (Stelwagen 2011). The polypeptide chains are modified in the Golgi vesicles through post-translational modifications (PTMs), where κ-casein is glycosylated, and the α-caseins and β-caseins are phosphorylated. The PTMs give the casein proteins different properties. (Holland 2008). The caseins in milk are hydrophobic phosphoproteins (Horne 2006), found in arranged micellar colloidal suspensions. The κ-casein, α-caseins and β-caseins are aggregated through hydrophobic interactions and linkage to colloidal calcium phosphate nanoclusters forming casein micelles (Fox & Kelly 2004; Giuffrida et al. 2017). The finished milk proteins are extracted from the mammary alveolar cells to the accumulation of milk in the alveolar lumen. A small proportion of the milk's proteins such as serum albumin, immunoglobulins, lactoferrin, and lacto-peroxidase are transported directly from the bloodstream to the milk collection by transport through or between the secretory cells without further processing of the proteins (Walstra et al. 2005; Stelwagen 2011). The synthesis of milk protein is a highly energy-intensive process. The amount of available energy thus affects the amount of protein formed in milk.

Microbial proteins form essential AA and constitute most of the protein being absorbed. The remaining proteins are taken up unaltered. Thus, the composition of the protein has less impact on the final protein presented in the milk synthesis. The amount of protein fed has a larger impact on the composition by increasing protein synthesis and protein yield. By increasing the availability of degradable carbohydrates in the feed could increase the high energy intensive synthesis of protein (Stelwagen 2011). Lactating cows have AA that are seen as essential when it comes to milk production. It usually includes methionine, lysine, and histidine found to be the most limiting AA in milk protein synthesis. A feed based on a high proportion of roughages has a low proportion of RUP, which could result in a deficiency of methionine. While a feed based on corn products as its RUP sources have lysine as its first limiting AA (Schwab & Whitehouse 2022).

Effects of milk proteins on milk properties

Milk contains caseins organised in casein micelles or whey proteins dispersed in the serum. At the pH of milk (6.8), the phosphorylated residues of the α_{s1}-CN, α_{s2}-CN and β-CN are highly ionized and bind well to divalent cations such as Ca^{2+} and colloidal calcium phosphate (CCP). The CCP have important function in building up the casein micelle as well as affecting the processing properties of milk (Walstra et al. 2005). The casein micelle is an arranged micellar colloidal suspension. It consists of a network of α_{s1}-CN, α_{s2}-CN and β-CN that are aggregated through hydrophobic interactions and linkages to CCP nanoclusters. By forming these bonds to cations causes a_{s1}-CN, α_{s2}-CN and β-CN to precipitate and neutralize their charges. The casein micelle core is surrounded by an outer layer of κ-CN which creates a steric hindrance keeping the colloidal structure together and inhibits precipitation. The α_{S1}-CN is found inside the micelle structure and is the most abundant casein in cow's milk. It has the largest proportion of phosphorylated serine residues and is calcium sensitive. The α_{s2}-CN differentiates from α_{s1}-CN casein by being slightly less phosphorylated and having cysteine residues forming disulphide bonds (Simpson et al. 2012).

The second most abundant casein protein is β-CN and is the most hydrophobic of the four phosphoproteins. It has the highest quantity of proline residues and lacks cysteine residue, resulting in low conformation of secondary structure of the protein. Some of the β-CN tend to leak out into the serum at lower temperatures, which affects the viscosity of the milk by increasing the density. This effect is reversible to some extent when temperatures increase again (Walstra et al. 2005). The κ-CN differs from the rest of the casein protein. Having less phosphorylated serine residues inhibits the κ-CN from precipitating due to weak binding to cations. The κ-CN creates a surrounding layer of the casein micelle by the look of tails around the calcium sensitive caseins inhibiting the caseins from precipitating in the milk (Fox & Kelly 2004; Walstra et al. 2005; Giuffrida et al. 2017). Its structure is stabilized by its cysteine residues creating intermolecular disulphide bonds, and it is the only glycosylated casein. Due to the glycosylation, the hydrophilicity of the κ-CN increase. The threonine's in the C-terminal of κ-CN carry oligosaccharides consisting of galactose, galactosamine and N-acetylneuraminic acid located in the end of the κ-CN tail (Fox & Kelly 2004; Walstra et al. 2005). Variations in the amount of κ-CN affect the casein micelle size (Walstra et al. 2005).

Dairy process and milk proteins

The standard basic procedure of processing the milk begins with a standardization of fat and protein content to avoid differences in processing and the quality of the final product. The second step is homogenization forcing the milk through small holes enforced by high pressure breaking the milk fat globules. Commercial milk is pasteurized to get rid of spoilage bacteria and inactivate enzymes responsible for affecting dairy products negatively. The heat treatment does not affect the casein micelles, but denatures the whey proteins at higher pasteurising temperatures and increases the casein micelles water absorption capability contributing to improved gel stability in processes of cheese and yogurt etc. (Simpson et al. 2012). At low temperature a part of the CCP dissolves, and the hydrophobic bonds in the micelle weaken, mainly causing β-CN to leak out and enabling proteolytic breakdown by enzymes in the serum. Increasing temperatures draw the β-CN into the micelle again stabilizing the micelle. At higher temperatures the concentration of CCP becomes higher, and at temperatures above 100C the κ-CN dissolves from the micelle (Walstra et al. 2005).

In fermented milk products such as cheese and yogurt, aggregation of casein micelles can be induced by rennet or acids, leading to coagulation changing the physiological properties. In cheese making, the rennet induced coagulation is caused by the enzyme chymosin found in abomasum of the calf. Chymosin cleaves the κ-CN from the micelle, reducing steric hindrance and negative charge of the micelle. The remaining para-casein aggregates by the calcium ion activity needed to form a binding network. Acid induced coagulation lowers the pH to 4.6 the isoelectric point of casein and reduces the net negative charges of the micelle by dissolving CCP and as a result the κ-CN tails fold towards the micelle reducing the steric hindrance and enabling aggregation of micelles (Walstra et al. 2005).

Milk fatty acids

Carbohydrate fermentation in the rumen releases the VFA: s acetate and butyrate, which are the main carbon source for *de novo* milk fat synthesis. The *de novo* synthesis accounts for around 60% of the total milk fat excreted. Most of the butyrate are converted to β-hydroxybutyrate either in the liver or directly at the rumen wall before being released to the bloodstream (Harvatine et al. 2022). The β-hydroxybutyrate and acetate are absorbed directly from the bloodstream to the mammary gland. These carbon sources are synthesized via acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in the secretory cells, forming short and medium fatty acids in lengths of 4 to 16 carbons. The large amounts of short chain fatty acids contribute to a lower melting range keeping the lipids in a liquid state (Walstra et al. 2005; Harvatine et al. 2022).

Longer chains of fatty acids from 16 C, 18 C and larger are absorbed from the digestive tract to the blood stream. Lipids absorbed from the digestive tract or from stored adipose tissue and arranged in triglycerides are carried by very-low-density lipoproteins (VLDL) in the bloodstream. These triglycerides carry predominantly longer (C16-C18) saturated fatty acids, small amounts of unsaturated fatty acids, odd and branched chain fatty acids, and some trans fatty acids (Harvatine et al. 2022). The walls of the mammary gland carry lipoprotein enzymes able to split the lipoprotein releasing the fatty acids and glycerol’s for uptake by the secretory cells. An enzyme, desaturase, are present in the secretory cell. Desaturase enables desaturation of stearic acid (18:0) to oleic acid (18:1), and in some cases change palmitic acid (16:0) to palmitoleic acid (16:1). The desaturation has lowering of melting range as an effect (Walstra et al. 2005). The released fatty acids from the different synthesis pathways are reconstituted in the cytosol with a glycerol assembling triglycerides. The triglycerides diffuse into lipid droplets increasing in size when transported towards the luminal cell wall. When reaching the secretory cell wall the droplets are encapsulated by the cell wall creating a protein membrane called a milk fat globule membrane around the droplets. The milk fat globules (MFG) are excreted by being snapped off the cell wall entering the alveolar lumen (Blowey & Edmondson 2010; Harvatine et al. 2022).The large amount of short chain fatty acids, desaturation, and variety of fatty acids in the triglycerides affects the rheological properties of milk by giving a liquid state of the milk fat and enabling the excretion of milk fat (Walstra et al. 2005).

The feed can shift the composition of milk fat through different synthetic pathways. A high ratio of stearic acid (18:0) in the feed results in larger amounts of unsaturated fatty acids in the milk due to the ability of desaturation. While a feed high in ratio of forages increases the amount of acetate reaching the mammary gland resulting in higher amounts of short to medium chain fatty acids (Walstra et al. 2005).

Fatty acid function in milk properties and dairy processes

The variation in TAG: s in MFG affects the rate of crystallization and the melting temperature in milk fat. The milk fat has a semi-solid character in room temperatures due to crystallization of TAG. The ability to crystallize affects the physical properties of milk fat and is used in the production of milk products high in fat such as butter and cream. The rate of crystallization is decreased in relation to increased amounts of components such as phospholipids, free fatty acids, and cholesterol. The composition of TAG also affects the crystalline process and digestibility. High concentration of saturated fatty acids gives higher melting temperatures than high concentrations of unsaturated fatty acids (Mattice et al. 2020).

Milk fat globules begin to form cream when stored cooled in a bulk tank to a limited extend, separating the cream from the skim milk due to immunoglobulins (agglutinins) attaching to the surface of the MFG. The MFGs intended for milk are homogenized, and then standardized. The sizes of the MFG are reduced by the homogenization process due to shear pressure. The process affects the milk fat globule membrane (MFGM), by infusing casein protein into the membrane reducing the aggregation properties of the MFG. Thus, reduces the risk of coalescence, cream separation, and increases milk scattering particles which increase density and the white colour of the milk. The process removes prooxidative components from MFGM reducing potential oxidative rancidity. The homogenization enables lipolysis due to TAGs being more available after the process. By heating milk after homogenization by pasteurization inhibits lipolysis through inactivation of enzymes (Simpson et al. 2012).

2.3 Environmental impact of milk production

Sweden's agricultural GHG emissions are primarily in the form of CH_4, carbon CO_2, and nitrous oxide (N_2O). The sector's total GHG emission is currently estimated to account for about 15% of Sweden's total GHG emissions. The main source of CO_2 comes from tillage, and energy consumption. Nitrous oxide (N_2O) is primarily released from manure management and nitrogen conversion in agricultural land. The majority of CH_4 derives from animal fermentative digestion. In total, CH_4 accounts for 42% of the total emissions from the agriculture sector in Sweden. In addition, emissions of minor air pollutants such as NH_3 occur, which contribute to acidification, eutrophication and deteriorate air quality (Naturvårdsverket 2022b). Excreta and manure can contribute to an excess of N leaking from the soil to the groundwater. The amount of N in the excreta is affected by the feeding of the animals and N uptake. Improving feeding management, nutrient uptake, tillage, and cultivation, reduces nutrient leakage and improves efficiency with financial gain (Pfeffer & Hristov 2005).

Agricultural practices also have a key role in curbing the GHG emissions. Methods such as cultivating lay have ecological functions as carbon sinks that bind CO_2, and at the same time reduce nutrient leakage from soils (Granström et al. 2022). The national climate goal in Sweden is to achieve net zero emissions of GHG emissions by 2045. Swedish agriculture aims to reduce GHG emissions, leakage of nutrients and at the same time increase food production, open landscapes, and biodiversity. A large part of the agricultural emissions come from natural biological processes, which affects the extent of mitigation potential without compromising production. By having other ecosystem services, the sector aims to contribute with net zero emissions by 2045 (Naturvårdsverket 2022b).

2.3.1 Enteric methane emissions

Within the rumen, a group of archaea called methanogens are present. They are special in a way that their anaerobic respiration has methane as a final metabolism product (Leahy et al. 2013). The archaea retain certain characteristic properties from their specific enzymes and coenzymes. These enzymes and coenzymes are essential in the process of reducing methyl groups in CO_2 and acetate to form CH_4 (Ferry 2010). The methanogens are for the most part hydrogenotrophic and use electron donors such as the hydrogen in the rumen as an energy source for their metabolism (Liu & Whitman 2008). Most hydrogenotrophic methanogens use H_2 as a primary energy source to reduce CO_2 into methane. Substrates such as CO_2 and H_2 can be generated from fermentation of sugar by anaerobic glycosylation through the Embden – Meyerhof – Parnas pathway. During the fermentation of glucose, NADH and pyruvate are generated. For the fermentation of sugar to continue,

NAD^+ needs to be regenerated, which is achieved by NADH then being oxidized to NAD^+ with CO_2 as the terminal acceptor in this case. The residues from the reaction are CO_2 and H_2 and lactic acid providing substrates for methane synthesis (McAllister et al. 2008).

A large part of the hydrogenotrophic methanogens also carry formate dehydrogenase (Fdh) and can use formate as their primary electron donor by oxidizing four formate molecules into one CO_2 and reducing it to methane (Liu & Whitman 2008). The methanogens can synthesize methane from three different types of substrates, which are CO_2, acetate, and methylated compounds. The CO_2 reduced by hydrogenotrophic methanogenesis undergoes seven steps where three specific coenzymes; methanofuran (MFR), tetrahydromethanopterin (H4MPT) and coenzyme M (CoM) carry the carbon to the final catalysing stage where CH_4 is formed. The second last step require cobalamins (B12) for the function of coenzyme M methyltransferase (Glasson et al. 2022). The last step includes the reducing enzyme methyl coenzyme M-reductase (MCR), which reduces methyl-CoM to CH_4 (Liu & Whitman 2008). These two last steps of methanogenesis have certain importance in reducing enteric methane production, further developed in the section of methane reducing feed additives.

The reduction of methylated compounds to CH_4 is terminated in the same manner in methanogenesis. This by the formation of methyl-CoM which is reduced by the key enzyme methyl coenzyme M-reductase (MCR) to CH_4 (Ferguson et al. 2000). Anaerobic oxidation of propionate and butyrate yields residues of acetate. Within the group Archaea in the rumen, only Methanosarcina and Methanosaeta are known to use acetate as energy to produce CH_4 as well as CO_2 and have it as a growth substrate. Acetate is modulated and divided into forming acetyl coA which then becomes CH_4 and CO_2 (Hobson & Stewart 1988). The formed methane diffuses out of the rumen to the lungs where it later exhales through the mouth and nose. The physiological function of methane formation is to extract the residual product and avoid the accumulation of H_2, which makes the methane the main H_2 sink, and keep low partial pressure of hydrogen in the rumen (McAllister et al. 2008). Of certain interest in research of methanogenesis is the methyl coenzyme M-reductase (MCR) that is found in the last step of all methanogenesis pathways forming CH_4. Targeting this enzyme has proven to open possibilities to affect the production of methane in the rumen (Beauchemin et al. 2020).

2.3.2 Financial and strategic plans to reduce environmental impact

Reducing GHG emissions is a global responsibility. The global climate agreement, the Paris Agreement, was established in 2016 with the aim of limiting global warming to a maximum of 2°C by mitigating GHG emissions. Climate research compiled by the UN's climate panel IPCC, forms the scientific basis for strategies to achieve the goals (Regeringskansliet 2020). The EU has a common strategic climate plan to contribute to the Paris Agreement, by reducing EUs total GHG emissions by at least 55 percent by 2030 compared to the levels of 1990. In addition, Sweden has its own stricter long-term climate goal of achieving net zero emissions of GHG emissions by 2045 (Naturvårdsverket 2022c). The strategy for achieving the environmental goals is continuously revised, and a new climate policy action plan is presented after a time span of 4 years (Naturvårdsverket 2022 c). The EU initiative includes the “European Green Deal”, a foundation to achieve a sustainable development of EUs agriculture. The new common agricultural policy (CAP) launching in 2023 is developed to be in line with the European Green Deal targets providing strategic plans and financial funds to the European farmers. New to CAP is the tool Eco-schemes, decided on national levels funding strategic plans contributing to the goals of the European Green Deal. Eco-schemes include funding of emission mitigation practices such as feed additives to reduce enteric methane production (European Commission 2021). Key factor in implementing a new cost in agricultural production.

A way to encourage the private sector to be a part of the green transition, is a collaboration between major associations: the UN, World Resources Institute (WRI), CDP Worldwide, and Worldwide Fund for Nature (WWF), developed an international initiative "Science-based target initiative" (SBTi). The collaboration aims to support the private sector, providing strategies on how companies can work to achieve the goals of the Paris Agreement (WRI 2022). Three of Sweden's largest dairies are all working to achieve the Paris Agreement goals with support from SBTi. Arla Foods' environmental goal strategy "The new 2030 target" has been approved, and Skånemejerier and Norrmejerier are waiting to have their strategies validated by the SBTi (Norrmejerier 2021; Skånemejerier 2021; Arla Foods 2022).

3. Method and materials

The study was divided into three parts covering a literature study, a laborative pilot study on the protein profile in milk samples, and two surveys on consumers' and dairy farmers' attitudes to methane reducing feed additives. The results from each section were summarized in a SWOT analysis describing the situation and the opportunities and challenges associated to methane reducing feed additives entering the dairy sector.

3.1.1 Literature study

A literature study was conducted on the additive's possible effects on rumen metabolism, milk properties and dairy processing, compiling literature on *in vitro* and *in vivo* trials with additives mitigating enteric methane production in dairy cows. The major question in this study was to investigate if methane reducing feed additives could pose changes in milk composition properties, and thus affect the dairy processing.

3.1.2 Laboratory study on milk protein profile from *A. taxiformis* fed dairy cows

A pilot study was conducted to characterize the protein profile in milk samples provided from a FORMAS funded project (2019-01266) by Rebecca Danielsson et al. (2021). In a feeding experiment, Swedish dairy cows were fed the additive red alga *A. taxiformis* during a period of 2 months. The study proceeded during 2020-2022, with feeding trials in the spring of 2022, to examine the effect on methane production, as well as the effect on the digestibility of feed (Danielsson et al. 2021). The trial included 30 cows divided into three groups fed different concentrations of *A. taxiformis*, and individual cow milk samples were collected every second week for analysis. The current study was conducted to investigate the possible impact on the milk protein profile and gain knowledge about effects that could affect dairy production when using milk from cows fed *A. taxiformis*. With the hypothesis that feeding *A. taxiformis* would have no effect on milk protein composition.

Milk samples from the group of cows (n=10) that received the highest inclusion rate of 0.3% organic matter (OM) of *A. taxiformis* was used in this report. Control milk samples (n = 10) collected before feeding trial (week 0), and milk samples (n = 10) from the last week of the feeding trial (week 8) were assessed in this study. Values for somatic cell count, and the concentrations of protein, lactose, and fat were obtained using Fourier Transform Infrared (FTIR) and used to evaluate significant differences in milk composition.

Materials and method for protein profile

The method used to characterize the milk protein profile was through capillary electrophoresis analyses, and the method used in this study where in line with Johansson et al. (2013) performed protein profile study. In short, this technique is used to separate proteins by running a liquid sample through an unfused silica standard capillary column, in this study 50 mm inside diameter, 40 cm active length (Johansson et al. 2013). Published studies of standard milk proteins retention time were used to recognize the peaks received from the capillary electrophoresis. Milk samples were collected at trial week 0 (control) and week 8 from the group of cows (n=10 for each group) that received the highest proportion of *A. taxiformis*, leading to a total of 20 individual cow milk samples. Sample buffer (SB) and run buffer (RB) were prepared according to description in Appendix 2.

Milk samples and buffer preparation:

A total of 20 samples of frozen milk were thawed in a 45°C water bath for 15 minutes until reaching room temperature. 200 µL of each sample was subsequently de-fattened by centrifugation for 10 min at 4°C and 10000 RPM. After removing the fat fraction, 400 µL of sample buffer (SB) (Appendix. 2) was added to the skim milk, and left standing at room temperature for 1 hour. The samples were filtered, and 30 µL of filtered sample was added to conic vials ready for being run in CE-MS.

Statistical methods

Mean values and standard deviations (SD) were calculated for each parameter. One-way ANOVA analysis of variance was performed in order to investigate if there were significant differences between the gross composition, SCC and protein profile ($p<0.05$). The software used for the statistical evaluations was Minitab® (version 19.2020.1, Minitab Inc., State College, PA, USA).

3.1.3 Surveys of consumers' and dairy farmers' attitudes to methane reducing feed additives to mitigate GHG emissions in the Swedish dairy

The third part of the study covered the attitudes to introduction of methane reducing feed additives into practice. Two surveys were conducted by LRF Dairy to investigate the attitudes / opinions among dairy farmers and consumers related to ruminants' environmental impact, and the introduction of methane reducing feed additives to reduce GHG emissions in the Swedish dairy sector.

The consumer survey conducted by Novus on behalf of LRF Dairy, took place with 1001 respondents in December 2021. The consumers were asked five questions about their views on ruminants' impact on the climate, and their attitude towards the use of additives reducing methane emissions in the dairy sector.

The producer survey conducted in December 2021 by LRF Dairy received responses from 117 dairy farmers from LRF Dairy's own Dairy Farmers panel (Sundin 2021). A substantially smaller group compared to the consumer group. The questions used in both studies are found in Appendix 3.

To compile the answers of the two surveys, eight questions were formulated (see below), summarizing the attitudes / opinions of consumers and dairy farmers of implementing methane reducing feed additives to the market. The formulated questions were used as a basis for the SWOT analysis, suggesting opportunities and challenges by additives entering the dairy sector. The statistical analysis was provided in the material from Novus, where statistically significant differences were calculated in advance and highlighted in the excel file.

Answers to the questions below are presented in the result section. Demographic factors dividing the consumers into groups included age, gender, geographic location, income, and education.

1. What type of consumer group is most positive to the use of methane-reducing feed additives, and why?
2. What are the consumers requirements regarding the use of methane reducing feed additives?
3. Is there a certain type of methane reducing feed additive more likely to be accepted by consumers?
4. What type of consumer group is currently opposed to use of methane reducing feed additives? Certain reasons for contradiction?
5. Do opinions differ between different geographical regions in Sweden? Are there differences between city and countryside?
6. How do producers view the use of methane reducing feed additives? What type of feed additives are producers most likely to choose for use?
7. Do farmers' have certain requirements regarding the use of methane reducing feed additives?
8. What are the possibilities for use, and what are the biggest contradictions?

3.1.4 SWOT analysis of the possibilities and challenges of implementing methane reducing feed additives in the dairy sector

A SWOT analysis was conducted based on the surveys of consumers' and dairy farmers' attitudes to methane reducing feed additives, but also including results from the literature study and the laboratory work, to summarize results with the objective to suggest opportunities and challenges of implementing future methane mitigating additives.

4. Results

The background covered ruminants feed metabolism, milk production, and enteric methane production. The most prominent methane reducing methods and feed additives will be evaluated in the following section.

4.1 Literature study

4.1.1 Chemically synthesized inhibitors

3-nitrooxypropanol (3-NOP)

Chemically synthesized inhibitors that inhibit methanogenesis and thus prevent production of methane in the rumen constitute an important area of research (Beauchemin et al. 2020). The chemical substance 3-nitrooxypropanol (3-NOP) is a precise additive targeting the active site of the nickel enzyme methyl CoM reductase (MCR) catalysing the last step in methanogenesis (Duin et al. 2016). It has been established that 3-NOP decreases the amount of CH_4 emitted, reducing enteric methane emissions with up to 30% in dairy cows (Lopes et al. 2016; Melgar et al. 2020a, 2021). Targeting methyl CoM reductase (MCR) with precision provides the opportunity to inhibit all methane producing pathways by Archaea's, which all require the targeted catalytic activity of MCR-enzymes to finalize their metabolism of methane (Duin et al. 2016). By inhibiting the hydrogen sink and formation of CH_4 with 3-NOP, an increase in emitted H_2 has been shown, in both *in vivo* (van Gastelen et al. 2020; Melgar et al. 2020a, 2021) and *in vitro* studies (Guyader et al. 2017). The fed 3-NOP decomposes in the rumen forming nitrite, nitrate and 1,3-propanediol (Duin et al. 2016). Studies have shown that there is no residual effect significantly altering the NH_3 or GHG emissions of manure from beef cattle fed 3-NOP, although long-term effects on nutrients accumulating in the soil need further studies (Owens et al. 2020, 2021).

Factors observed to have a negative effect on the extent of reduction of emitted CH_4 from 3-NOP include a too low dosage of 3-NOP fed (Dijkstra et al. 2018; McGinn et al. 2019), or a high dietary fibre content (Dijkstra et al. 2018). Feed ration are thought to affect the amount of methyl-coenzyme M in the ruminants. A ration rich in fibre are thought to increase concentration of methyl-coenzyme M, thus require an increased amount of 3-NOP to increase mitigation potential of the additive (Dijkstra et al. 2018). Dairy cows have a greater feed intake and feed conversion compared to other ruminants, resulting in more fermentative products. This was proposed to result in a relatively lower concentration of methyl-coenzyme

M, due to multiple pathways for hydrogen sinks, thus increasing the potential effect of 3-NOP targeting methyl CoM reductase (MCR) (Dijkstra et al. 2018). The additive can be supplemented in pellet form, powder form mixed with total mixed ration (TMR) or as a top-dress. The effect has been reported to be equivalent regardless of the form in which the additive was given. Offering the supplement evenly throughout the day gave better results than feeding occasionally (Reynolds et al. 2014; Van Wesemael et al. 2019).

The European Food Safety Authority (EFSA) carried out a comprehensive scientific assessment on the safety of the use of the additive Bovaer® with the active substance of 3-NOP in dairy production. Experts in The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), concluded that Bovaer® has the potential of reducing enteric methane emission. The additive is safe for usage in dairy and other ruminant production, and it does not pose a health risk for either humans, animals, or the surrounding environment. EFSA concluded that feeding the additive in 60 mg/kg DM feed had no effect on performance (EFSA et al. 2021). In February 2022, the marketing of 3-NOP was approved by the EU member states and the additive was available on the market within months. The additive is the first of its kind available on the EU-market (European Commission 2022a).

Regarding the impact of feeding 3-NOP on milk production and the composition of the milk recent studies have shown inconsistencies in the results. When incorporating the additive 3-NOP into the feed ratio according to the EFSA recommended daily intake of 60 mg/kg feed DM (EFSA et al. 2021), studies have shown both an increase (Lopes et al. 2016; Melgar et al. 2020b, 2021), and no effect (Melgar et al. 2020a) on milk fat yield and concentration. In two studies by Melgar et al, results showed an increase in the amount of *de novo* synthesized short fatty acids (C4-C6) (Melgar et al. 2020a) and (C6-C8) (Melgar et al. 2021). In contrast, the total amount of VFA was reported to decrease, whereas the total milk yield was unchanged (Melgar et al. 2020a). Lopes et al., (2016) instead reported that the total milk fat concentration increased after using 3-NOP in dairy cows, and the total VFA was unchanged (Lopes et al. 2016). The molar proportion of VFA shifted, with increasing levels of butyrate, propionate, valerate, and isovalerate (Lopes et al. 2016) while acetate, in contrast decreased (Lopes et al. 2016; Melgar et al. 2020a), resulting in a decreased acetate-to-propionate ratio (Lopes et al. 2016). Melgar et al (2021) also reported a reduction in trans fatty acid (cis-9, trans-11 CLA) possibly due to a shift in the VFA with increasing levels of valerate and butyrate (Melgar et al. 2021). Concentration and yield of lactose was unchanged (Reynolds et al. 2014; Melgar et al. 2020b; a, 2021). In contrast, Hirstov et al (2015) reported an increased yield of lactose. In the same study, the total milk protein yield also increased without changes in milk yield (Hristov et al. 2015). In the studies with unaffected lactose yield, 3-NOP had no effect on the concentration or yield of milk protein

(Reynolds et al. 2014; Melgar et al. 2020a; b, 2021). The double intake of the recommended intake has shown effects such as a reduction in both digestibility, energy supply, and reduced acetate concentration (Reynolds et al. 2014).

Dicarboxylic acids

Another method used to reduce methanogenesis is by competition for H_2 in the rumen by stimulating the growth of other H_2 utilizing bacteria strains. By feeding dicarboxylic acids in the form of fumarate, malate or aspartate as additive, functioning as electron acceptors in the succinate–propionate pathway, an increased propionogenesis has been observed (Jouany & Morgavi 2007). Addition of the dicarboxylic acids will benefit bacteria such as *Fibrobacter succinogenes*, and *Selenomonas ruminantium* ssp, utilizing the dicarboxylic acids with H_2 as an energy source. Reducing fumarate to form propionate (Asanuma et al. 1999), is a more thermodynamically favourable pathway compared to methanogenesis (Sejrsen et al. 2006). By favouring propionogenesis, there will be less H_2 available for the methanogens in the rumen (Asanuma et al. 1999). Adding fumarate has shown a reduction potential in CH_4 ranging between 0-38%. Adding reducing agents, such as fumarate or malate, resulted in formation of propionate and butyrate, although all added precursors did not engage in propionate/butyrate formation; some of the added dicarboxylic acid was converted into acetate affecting the mitigation negatively (Sejrsen et al. 2006).

Nitrates

A compound found competitive as an alternative hydrogen sink is the inorganic anion nitrate (NO^{3-}). The two reduction steps of nitrate to form ammonia competes with the amount of CO_2 reduced to CH_4 (EFSA et al. 2020). Nitrate can be used as an additive to improve animal productivity, where the feed is low in protein. The methanogenic properties decrease with high quality forage and concentrates (Gerber et al. 2013). Feng et al (2020) showed the effect of nitrate supplementation to be dose dependent and greater in dairy cows than in beef steers. This is due to the larger concentrations of VFAs and H_2 in the dairy cow rumen receiving a higher feed intake (Feng et al. 2020). Nitrite reductase activity forming ammonia is lower than the conversion of exogenous nitrate to nitrite. Causing an excess of nitrite in the rumen that can diffuse into the bloodstream. Large amounts of nitrite can cause a toxic effect in the form of methemoglobinemia, where the blood becomes deoxygenated because of the formation of methaemoglobin. A gradual adaptation to larger amounts of nitrate in the feed can be achieved. Due to these health concerns, EFSA has concluded a limit value for ruminant intake of nitrate, i.e., not more than 64 mg nitrate / kg body weight (bw) per day to reduce the risk of toxic levels for the animals (EFSA et al. 2020). Products from animals fed additives according to this limit value are considered not to pose any concern for human health (Authority (EFSA) 2009)

4.1.2 Natural raw materials as methane reducing feed additives

Red algae additives

Seaweed algae, in addition to their ability to produce bioactive compounds, provide a source of nutrients, such as carbohydrates, lipids, proteins, and nucleic acids (Richmond & Hu 2013). Most strains of seaweed have shown to reduce enteric methane production to various degrees by affecting the rumen fermentation (Machado et al. 2016). According to Machado et al., (2014) the most promising and effective seaweed species in the reduction of enteric methane are the marine red macroalga *Asparagopsis* and the brown macroalga *Dictyota*, with potential of reducing enteric methane output by 98.9% and 92.2% respectively (Machado et al. 2014). Their effectiveness in reducing methane was shown to be due to a shift in the rumen fermentation, increasing the molar concentration of propionate and reducing the total VFA concentration (Machado et al. 2014).

The macro algae red seaweed *Asparagopsis spp.* can inhibit formation of methane through synthesizing halogenated metabolites by oxidation of chloride, bromide, and iodide with halo peroxidases (Machado et al. 2016). Bromoform (CHBr3) is the most abundant organic halogenated compound and is thought to be the main driver of the antimethanogenic activity in *Asparagopsis* (Machado et al. 2016). These secondary metabolites act as a chemical defence mechanism in their host organism through their high biological activities. Their active metabolites are often found in high quantities in red algal populations and kelp forests, among others (Paul & Pohnert 2011). The halogenated compounds inhibit the cobamide-dependent methyltransferase step required in methanogenesis by reacting with reduced cobalamin (B12) necessary for the enzymatic activity decreasing the enzyme activity and thus the formation of methane (Wood et al. 1968). Bromoforms are hazardous in large quantities and are classified "likely to be carcinogenic to humans" by U.S. Environmental Protection Agency (EPA) (2018). The halogenated compounds are therefore regulated to the limit value 81 µg / L in drinking water (US EPA 2018). Muizelaar et. Al (2021) reported values of 6-35 µg/L of CHBr3 measured in milk from a feed trial adding *A. taxiformis* with inclusion rates ranging between 67-333 g/DM containing 1.26 mg/kg DM of CHBr3. Values ranging between inclusion rates of 0.24, 0.44, and 1.34% of the feed organic matter (OM). The values varied in each group during the feeding trials. The measured values of CHBr3 in comparison to the water's maximum values set by EPA of 81 µg / L (US EPA 2018), the measured values contributed up to 44% of the maximum value for water (Muizelaar et al. 2021).

In addition, red algae, including *Asparagopsis,* have a wide spectrum and amount of other bioactive properties (Salvador et al. 2007). In a study by Salvador et al., taxes *Bonnemaisonia asparagoides* and *B. hamifera* were observed to have a particularly high antimicrobial action against Gram-positive bacteria and yeast. The species of the red algae *Falkenbergia rufolanosa* and *Asparagopsis armata* also showed a high activity against Gram-negative bacteria. Depending on the season, the algae produce different amounts of bioactive compounds and the amounts of bioactive compounds can therefore differ during the year (Salvador et al. 2007).

An *in vivo* study, with supplementation of *A. armata* at an inclusion rate of 0.5% and 1.0% of the diets organic matter to dairy cows showed a decrease of 26.4% and 67.2%, respectively, in methane production. The 1.0% inclusion rate showed reductions in both milk yield and protein yield and a decrease in DMI compared to the control. The low inclusion rate had no effect on DMI or milk yield. The study observed no significant increase in bromoform in the milk at either inclusion rates (Roque et al. 2019). Muizelaar et al. (2021) reported an increase in milk fat concentration in higher inclusion rates of *A. taxiformis* thought to be an effect of a negative energy balance and increased uptake of fat from body reserves to the milk from a reduced DMI (Muizelaar et al. 2021). Another *in vivo* study supplementing *A. taxiformis* experienced lower DMI due to issues where cows avoided the feed with the additive, especially at the higher inclusion rates. The observed effect on milk yield in an *in vivo* study on *A. taxiformis* was an increase by 15.6% with low inclusion, and a reduction with medium to high inclusion of the additive (Muizelaar et al. 2021). An *in vitro* study with *A. taxiformis* showed no significant effect on total VFA or degradability of substrates with an inclusion rate of 2.0% OM. An inclusion rate over 5.0% OM (Kinley et al. 2016), or concentration of bromoform larger than 25 μM showed a significant decrease in total VFA (Machado et al. 2016).

Like other mitigation practices, the amount of acetate decreases while mainly propionate and to a lesser extent butyrate increases with the higher inclusion rates of additives, following the reduction in methane (Kinley et al. 2016). The availability of *A. taxiformis* is today a concern as it is not yet produced commercially (Honan et al. 2021).

Natural plant secondary compounds - Tannins, saponins and essential oils

Plant materials such as leaves, roots, seeds, and flowers contain a variety of phytocompounds. These secondary metabolites are often specific to the plants, develop depending on the environmental conditions, and have varying functions to benefit the plant. They may have impact on colour, odour, protection against viruses, bacteria and moulds (Balandrin et al. 1985), and have potential anti methanogenic properties when fed as an additive. Plant bioactive components that are believed to have potential to inhibit methanogenesis include compounds e.g., tannins, saponins and essential oils. The composition and concentration of phytocompounds in the essential oils can vary depending on various factors such as environmental factors, time for harvest, utilization process, and storage (Patra et al. 2012; Hernandez et al. 2017; Dhanasekaran et al. 2020).

Tannins are found in both forage and concentrates in various amounts, either in condensed or hydrolysable form. Tannins contain phenolic hydroxyl groups that can form insoluble complex structures hard to digest, which may affect nutrient utilization in feed, by reducing the amount of rumen degraded protein. This can lead to decreased amount of NH_3 left from microbial protein metabolism, reducing the amount of NH_3 excreted with the urine (Patra et al. 2012). Tannin's bacteriostatic properties are thought to influence methanogenesis through inactivation of enzymes inhibiting MO growth, proposed to have the potential of reducing 60% of enteric methane produced (Tavendale et al. 2005; Patra et al. 2012; Dhanasekaran et al. 2020). Decrease in OM digestibility reducing milk fat and protein yields have been reported when fed a large inclusion rate of tannins.

Saponins are amphiphilic glucoside molecules having its biological activity linked to affecting the integrity of cell membranes giving properties as suppressing protozoa and bacteria or enhancing nutrient uptake (Alamgir 2018), though its functions inhibiting methanogens is not clearly understood if it is thought to decrease availability of H_2 in the rumen reducing methanogenesis (Patra & Saxena 2010). Variation in effect on OM digestibility, and a methane reduction potential of 6-27% have been reported.

The effect of essential oils (EO) in *in vivo* studies has shown varying results on both reduction of methanogenesis, and effect on milk properties. EOs have diverse properties affecting microbes differently (Cobellis et al. 2016). *In vivo* studies have used combinations of different EOs to improve digestion and reduce methanogenesis by targeting the archaea. *In vitro* studies showed reduction in abundance of protozoa and archaea, resulting in a reduction of methanogenesis (Cobellis et al. 2016). EOs effect on ruminants are affected by factors such as stage of lactation, mixture of EO supplemented, amount of EO given and time (Tassoul & Shaver 2009; Santos et al. 2010; Elcoso et al. 2019). A mixture of coriander essential oil, eugenol, and geranyl acetate feed as supplement resulted in an increase in feed efficiency shown in increased milk fat synthesis (Santos et al. 2010), milk solids and yield, and a reduction in methane concentration after 4 weeks of supplementation (Elcoso et al. 2019). Though a reduction of body condition gain was reported in the case with increased milk fat synthesis (Santos et al. 2010). While other studies saw no effect of the supplemented EOs on the milk yield (Tassoul & Shaver 2009; Elcoso et al. 2019). A sensory evaluation was performed by Silva et al. (2020), with milk from a trial with a mixture of the EOs: carvacrol, cinnamaldehyde, eugenol, and capsaicin supplemented. Of the 63 who tested the milk samples, 59% of the consumers were able to distinguish the milk from the group of cows that received EOs (Silva et al. 2020).

Similar for tannins, saponins and EOs are the insufficient scientific basis to conclude the CH_4 mitigating effect, especially in long term exposure that can lead to an adaptation of the rumen microbiota reducing the antimethanogenic effect (Gerber et al. 2013).

4.2 Laboratory pilot study on milk composition and milk protein profile in milk from cows fed *A. taxiformis*

4.2.1 Composition of milk from individual cows fed *A. taxiformis*

The results from the FTIR analysis of milk composition and cell count were obtained from Danielsson et al. (2021). Values were evaluated using the software Minitab® to perform One-way ANOVA, and differences were considered significant if p-value ≤ 0.05.

There were no significant differences in amount of fat, protein, or lactose content (table 1) between control week samples (W-0), and samples from 8 weeks (W-8) with the highest inclusion rat of *A. taxiformis* added to the diet.

Table 1. Results from one-way ANOVA test for the contents of all types of fat, protein, lactose concentration (%) and somatic cell count (SCC 10^3/mL) in milk samples (n=10 from week 0 respectively week 8) from dairy cows fed 0.3% organic matter of A. taxiformis. Differences were considered significant if p≤ 0.05

Milk Components in % and SCC (10^3/mL)	*Mean W-0*	StDev *W-0*	*Mean W-8*	*StDev W-8*	*P-Value*
Fat	4.19	0.59	4.31	0.56	0.64
Protein	3.50	0.25	3.64	0.28	0.26
Lactose	4.81	0.11	4.77	0.13	0.44
SFA	2.75	0.46	2.82	0.36	0.72
UFA	1.30	0.22	1.22	0.16	0.37
MUFA	0.97	0.19	0.90	0.13	0.35
PUFA	0.14	0.03	0.13	0.03	0.45
C16:0	1.12	0.20	1.21	0.18	0.31
C18:0	0.67	0.15	0.55	0.10	0.05
C18:1C9	0.77	0.15	0.73	0.10	0.53
C14:0	0.51	0.07	0.50	0.06	0.83
SCC (10^3/mL)	65.3	85.8	198	458	0.38

4.2.2 Milk protein profile in milk from cows fed *A. taxiformis*

The results from the characterization of milk protein composition using CE, were analysed through the software Minitab® by a One-way ANOVA, and differences were considered significant if p-value ≤0.05 (table 2).

Table 2. Results from one-way ANOVA test for the relative concentration of the total protein detected milk protein profile (%) and pH in milk samples from dairy cows (n=10) fed 0.3% organic matter of A. taxiformis. Differences were considered significant if p≤ 0.05. Significant differences between W0 and W8 are indicated in bold.

Relative concentration of the total protein detected milk protein profile (%)	*Mean W-0*	StDev W-0	*Mean W-8*	*StDev W-8*	*P-Value*
α_{s1}-CN	29.35	2.12	28.70	1.22	0.42
α_{s2}-CN	7.48	1.14	6.94	0.83	**0.03**
κ-CN	4.96	1.87	2.00	1.49	**0.001**
ß-CN	4.25	2.51	5.19	0.54	0.29
$ß_{A1}$-CN	21.26	1.24	23.31	7.41	0.52
$ß_{A2}$-CN	29.51	9.62	26.02	10.98	0.46
α-LA	2.15	0.69	2.05	0.74	0.77
ß-LG	6.69	1.89	6.50	2.49	0.85
Total ß-CN	43.50	3.93	49.34	2.86	**0.001**
Total whey (α-LA, ß-LG)	8.84	2.50	8.55	3.19	0.83
Total CN	85.75	5.95	86.44	1.55	0.73
pH	6.73	0.09	6.74	0.14	0.96

The relative concentrations of the proteins α_{s2}-CN, κ-CN, and total β-CN differed significantly in milk from week 0 (control) and week 8, respectively. The results showed a significantly higher mean of α_{s2}-CN, and κ-CN in samples from week 0 compared to the samples of week 8. The mean of total β -CN was significantly higher in week 8 compared to week 0. These results suggested decreasing relative concentrations of α_{s2}-CN and κ-CN, while increasing total β -CN when *A. taxiformis* was added to the feed during an 8-week period (table 2).

4.3 Surveys of consumers' and dairy farmers' attitudes to methane reducing feed additives to mitigate GHG emissions in the Swedish dairy

Answers from the producer and consumer surveys were summarized. Demographic factors dividing the consumers into groups were based on age, gender, geographic location, income, and education. Significant differences were calculated by NOVUS and used in the current study when answering the questions summarizing the attitudes / opinions of consumers and dairy farmers. Figures 1 and 2 summarize the answers received in both surveys.

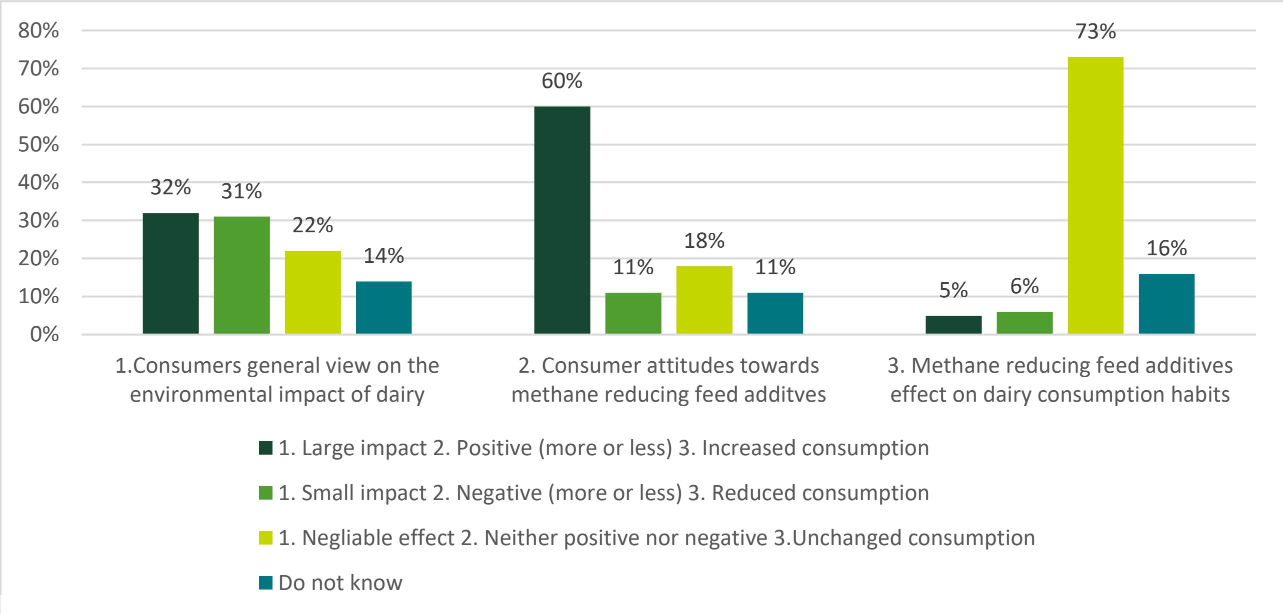

Figure 2. Answers in the consumer survey on consumers' view (n=1001) on dairy environmental impact, use of methane reducing feed additives, and effect on dairy consumption habits by usage of methane reducing feed additives. Source: NOVUS Consumer survey (2021). The numbers after each coloured box under the staples represent the different answers for each of the three questions.

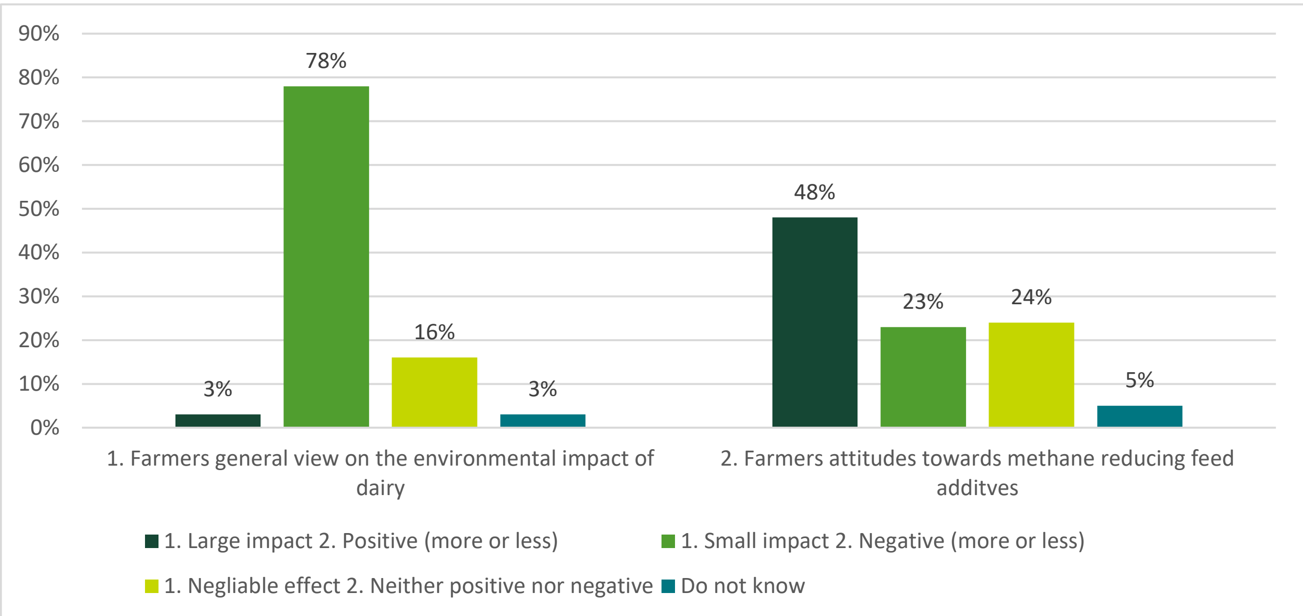

Figure 1. Answers from the dairy farmer survey on farmers' view (n=117) on dairy environmental impact, use of methane reducing feed additives. Source: Results Dairy Farmer Panel survey (2021) by LRF Dairy. The numbers after each coloured box under the staples represent the different answers for each of the two questions.

The perception of the environmental impact of ruminants differed between consumers and producers, where 78% of the dairy producers considered the impact to be less prominent, compared to 31% among the consumers, (Table 1 and 2). Consumers (60%) were to a larger extent more positive to the use of methane reducing feed additives compared to the farmers (48%). The group opposed to the use of these additives were larger in the group of farmers (23%) compared to the group of consumers (11%).

4.3.1 Questions summarizing the attitudes of consumers and dairy farmers of implementing methane reducing feed additives in the market based on answers compiled in two surveys.

1. *What type of consumer group is most positive to the use of methane-reducing feed additives, and why?*

   Men were significantly more positive than women to the use of methane reducing feed additives. The age group 18-29 was significantly more positive than the groups of 50-64- and 65–79-year-olds. Consumers living in cities were significantly more positive than consumers living in the countryside. The level of education in the group of interviewed consumers was high, with university studies being significantly more common than just primary school. Overall, those with children living at home were significantly more positive than those with no children. Households with a yearly income of >800tkr were significantly more positive compared to households with an income of >300tkr/year. Overall, all regions in Sweden were more positive than negative to supplementing dairy cows with methane reducing feed additives. The respondents' consumption patterns were expected to increase significantly with the use of methane reducing feed additives in the group of 18–29-year-old, compared with older age groups. However, the vast majority would keep their consumption unchanged.

   The group of consumers positive to additives had a desire to continue consuming meat and dairy products and recognised the benefits of having ruminants in ecosystem services and agriculture. The respondents believed that all industries have a responsibility to reduce their climate impact. The use of a well-studied additives that can reduce the negative environmental impact of the dairy industry was seen as one option to improve dairy production.

2. *What are the consumers requirements regarding the use of methane reducing feed additives?*

   The most prominent consumer request was that animal welfare must not be compromised for a reduced environmental impact. That was raised both from the groups that were positive to the use of additives, but also from the groups who were unsure or opposed to the use of these types of additives. The additives should be well studied to guarantee no negative consequences for the animals, the environment, or the economy in the long run. The positive group of respondents could see a risk that the pricing of the dairy products could be slightly increased.

3. *Is there a certain type of methane reducing feed additive more likely to be accepted by consumers?*

   Consistent in all response groups was that the consumers emphasized the importance of having the milk production process as natural as possible. The largest response group, 60%, was positive to the use of the additives but preferred to see the use of natural raw materials. The use of algae as a natural additive was raised by one respondent as an example.

4. *What type of consumer group is currently opposed to use of methane reducing feed additives? Certain reasons for contradiction?*

   Mainly women in the 50–64- age group were more opposed to the use of methane reducing feed additives. The age group of 50-79 was significantly more negative to an additive than the 18-49 age group. There were significantly more respondents that were opposed to the use of additives in the group with only high school education than in the group with university education. A significantly larger group of consumers in Småland compared to Stockholm was opposed to using feed additives. In the response groups with a negative attitude towards using additives, it was mainly because this consumer groups believed that cows' impact on the environment was negligible, and therefore they could see no need for additives to reduce the ruminants' environmental impacts. The respondents were against using unnatural additives since it was thought to affect animal welfare in a negative way.

   Consumption patterns would decrease significantly with the use of methane-reducing additives in the older age group of 50-79, compared to the younger age groups. Women would reduce their consumption of dairy products significantly more than men. However, the majorities among men and women would not change their consumption habits if methane-reducing additives were used.

5. *Do opinions differ between different geographical regions in Sweden? Are there differences between city and countryside?*

   The consumer survey distinguished opinion differences on the additives associated with consumers view on the extent of climate impact of ruminants. Differences were observed between consumers living in the city and consumers in the countryside. A significantly larger proportion of the consumers in large cities thought that the environmental impact of cows was significant, compared to consumers in smaller cities. In urban areas and rural municipalities, a significant proportion looked upon the ruminants' impact on the environment as small. At a regional level, a significant proportion of respondents from Stockholm considered cows to have a larger' effects on the climate rather than a small effect.

6. *How do producers view the use of methane reducing feed additives? What type of feed additives are producers most likely to choose for use?*

   The attitudes among dairy farmers to the use of methane reducing feed additives, showed that 48% were positive, 23% were negative and the remaining 29% were neither positive nor negative or had no opinion on its use. The farmers expressed will and positive attitude to reduce the environmental impact of the sector but were sceptical whether changing the cows' metabolic processes should be a major area of focus. The issue of financing the additives was raised due to small margins for additional expenditures for the farmers. Financial compensation from authorities or increased product prices for consumers were seen as alternatives. Around 80% of the producers believed ruminants to have little to very little impact on the environment, and 16% of the producers were of the opinion, that cows had no major or minor impact on the climate. A small proportion (3%) considered ruminants to have a large or very large impact on the environment and the remaining 3% answered that they did not know. In general, the producers thought that the focus on the impact of ruminants in the climate debate is not fair. Most producers see ruminants as part of the natural cycle, with more ecosystem services and benefits to society than it has negative impact on the environment. The need for a product with a methane-reducing effect is thus seen as a response to public opinion, and additives as something superfluous for the most part. There are other potentials associated with other aspects of dairy production to reduce the environmental impact of farming, such as improving feed production or transport. No specific type of additives was mentioned in the producers' answers, other than one producer who refers to "If the additives were something chemical, I am negative about it. If it was biochar or similar, I am only positive about it." This could be an indication, that dairy producers want to keep production as similar as it is today.

7. *Do farmers' have certain requirements regarding the use of methane-reducing additives?*

   The farmers' requirements for the additives are mainly that they are well-studied and safe for use by the animals and above all that the cost of additives does not become the farmer's responsibility. It should either be financed through financial agricultural support or paid by society.

8. *What are the possibilities for use, and what are the biggest contradictions?*

   The attitude towards the use of methane-reducing additives was predominantly positive in both the consumer and producer surveys. The curiosity for improving dairy production and reducing its impact on the environment is a fact. However, if the inhibition of enteric methane production is the best way to do it, there are divided opinions about, especially among producers.

   The largest contradiction to use of additives was among the consumers who did not see additives as something necessary and did not want to see the cows to have something unnatural in their diet. In groups expressing that ruminants have a minor impact on the environment, especially among dairy farmers, the will to use the additives was negatively affected, and requires a guarantee of financial benefit for the producers and no negative effect on the animals.

4.4 SWOT analysis of the possibilities and challenges of implementing methane reducing feed additives in the dairy sector

The summaries of answers related to the questions in the consumer and producer surveys, pilot study, and the results from the literature study, were used in two different SWOT analyses for chemically synthesised feed additives, and plant derived feed additives, respectively. Possibilities and challenges associated with each type of additive were evaluated when considering their possible use in dairy production.

4.4.1 SWOT analysis of chemically synthesised methane reducing feed additives

Figure 3. SWOT analysis associated to the use of chemically derived enteric methane mitigating additives (3-NOP, dicarboxylic acids, nitrates) in dairy production. Results collected from findings in the literature study, laborative study and the consumer and producer surveys.

References:[1.] (Jouany & Morgavi 2007) [2.] (EFSA et al. 2020) [3.] (EFSA et al. 2020) [4.] (Lopes et al. 2016; Melgar et. al. 2020a, 2021) [5.] (European Commission 2022a) [6.] (Naturvårdsverket 2022c) [7.] (Consumer survey. 2021) [8.] (Producer survey. 2021) [9.] (European Commission 2021) [10.] (Dijkstra et al. 2018) [11.] (EFSA et al. 2020) [12] (Gerber et al. 2013) [13.] (Salvador et al. 2007) [14.] (Melgar et al. 2021).[15.] (Dijkstra et al. 2018)

4.4.2 SWOT analysis of plant derived methane reducing feed additives

Figure 4. SWOT analysis of plant derived enteric methane mitigating additives (algae, plant secondary metabolites) in dairy production. The results are collected from findings in the literature study, laborative study and the consumer and producer surveys.

References: [1] (Honan et al. 2021) [2.](Muizelaar et al. 2021) [3.](Gerber et al. 2013) [4.] (Kinley et al. 2016) [5.] (Current reports laboratory study) [6.] (Salvador et al. 2007) [7.] (Silva et al. 2020) [8] (Roque et al. 2019)[.9.](Consumer survey. 2021) [10.](Producer survey. 2021) [11.](Naturvårdsverket 2022c) [12.] (European Commission 2021)

5. Discussion

Enteric methane from livestock is a significant contributor to the total global agricultural GHG emissions (Gerber 2013). Recent mitigation strategies to reduce methane emissions in livestock production are through additives fed to alter the rumen metabolism, inhibiting the formation of CH_4 (Beauchemin et al. 2020). The purpose of the study was to evaluate potential effects on use of the most prominent methane reducing feed additives in dairy production. This study focuses on 3-nitroxypropanol and the red algae *Asparagopsis spp* (Beauchemin et al. 2020).
The study aimed to investigate possible changes in milk composition and milk quality properties, that could affect dairy processing from the use of methane mitigating additives for dairy cows. Together with a compilation of consumers 'and producers' attitudes towards the use of methane-reducing feed additives. This to evaluate the opportunities and challenges with implementing methane reducing feed additives in dairy production.

Plant derived and chemically synthesised additives have both the potential of mitigating around 30% of the total enteric methane without reducing DMI and milk yield. This effect results from shifting the molar proportion of VFA by reduced acetate-to-propionate proportion and increased butyrate (Gerber et al. 2013; Lopes et al. 2016; Roque et al. 2019; Melgar et al. 2020a, 2021). An even higher mitigating effect could be achieved but only with the concomitant effect of decreasing VFA, DMI and milk yield. Unwanted effects have been observed with both algae (Kinley et al. 2016; Roque et al. 2019), 3-NOP (Reynolds et al. 2014), and tannins (Gerber et al. 2013) when fed as additives at high ratios or exceeding the recommended dose. The DMI and milk yield are suppressed with higher doses of additives, and high methane mitigating levels. This suggests that their potential of mitigating enteric methane is dose dependent and has a limit, which when exceeded, will result in a decreased nutrient utilization and milk yield.

Chemically synthesised additives

During the time period of this study, a 3-NOP additive, i.e. Bovaer® 10 (DSM Nutritional Products Ltd) (EFSA et al. 2021), was approved for use in EU livestock production (European Commission 2022a). The active substance 3-NOP targets the last catalytic step in methanogenesis by inhibiting methyl CoM reductase (MCR) (Duin et al. 2016). When 3-NOP was fed at the recommended daily intake of 60 mg / kg feed DM (EFSA et al. 2021), *in vivo* studies showed increased amounts of H_2 emitted (Melgar et al. 2020a, 2021; van Gastelen et al. 2020), a decrease in the trans fatty acid 18:1 (Melgar et al. 2021) and increased *de novo* synthesized SCFA (C4-C8) (Melgar et al. 2021: Melgar et al. 2020a). At the same time, the ratio acetate-to-propionate decreased (Lopes et al. 2016), and the concentration of butyrate and propionate increased (Melgar et al. 2021). The precursor for milk

SCFA is acetate and butyrate, where butyrate is precursor in *de novo* synthesised C4-C8 (Walstra et al. 2005; Harvatine et al. 2022). The observations are thus indications of a shift in FA pathway by increasing *de novo* synthesised FA. Whether or not the increased concentration of SCFA in the milk fat triglycerides also affect the processing properties of the milk fat, such as the melting range, need further studies to conclude (Mattice et al. 2020). Despite the increased concentration of propionate, i.e. the precursor in gluconeogenesis (Janssen 2010), no increase in milk lactose and protein yield or concentration was reported (Reynolds et al. 2014; Melgar et al. 2020a; b, 2021).

The potential mitigating effect of 3-NOP was proposed to be reduced by a feed ration high in fibre content, probably caused by increased concentration of methyl coenzyme M in the rumen (Dijkstra et al. 2018), something to be taken into consideration in Sweden where a high intake of forages is common. Research on the long-term effects of exposure to 3-NOP and its residual nitrogen products is needed to ensure that nitrogen residuals from 3-NOP do not contribute to excess N_20 emitting from the manure (Owens et al. 2020, 2021) or causing toxic levels of nitrite accumulating in the rumen (EFSA et al. 2020).

Nitrate diets are more suitable as a supplement in areas where an unbalanced feed ration needs increased nitrogen content to improve animal productivity, rather than function as a methane reducing feed additive (Gerber et al. 2013). However, extra control of the roughage is required as the amount of nitrate in the feed varies, and the risk of nitrate poisoning can then occur. The methane mitigating potential of nitrate is reduced with a sufficient feed ratio and rather pose a risk of being over fed and cause animal health issues or being excreted and not being cost effective.

Natural plant derived additives

The macro algae red seaweed *Asparagopsis spp* are the most promising plant derived methane reducing feed additives (Machado et al. 2014). They have the potential of reducing enteric methane production around 27% with low inclusion rates, e.g., 0.5% diet OM, without comprising DMI, VFA, or milk yield (Roque et al. 2019). Like the other mitigating strategies, studies report that the molar concentrations of VFA shifted by increasing propionate and reducing acetate (Kinley et al. 2016). The active methane mitigating compound in *Asparagopsis spp* is bromoform (CHBr3) (Machado et al. 2016), which by reacting with reduced cobalamin (B12), inhibits the cobamide-dependent methyltransferase in methanogenesis (Wood et al. 1968). Muizelaar et al. (2021) concluded that CHBr3 can be excreted through the milk and reported varying amounts of CHBr3 in milk after feeding *A. taxiformis*. Levels of concern could be reached in areas where water already contains a substantial amount of CHBr3, by exceeding the set maximum values for water (Muizelaar et al. 2021). In contrast, other studies found no increase in $CHBr_3$ at inclusion rates ≥1.0% OM (Roque et al. 2019), and further studies are needed to conclude factors affecting $CHBr_3$ excretion to the milk.

The laboratory part of this study on the effect of *A. taxiformis* (0.3% OM) on milk protein profile showed no effect on the total concentration of protein or the total gross composition. There was, however, a significant reduction in the relative concentrations of α_{s2}- and κ-casein and an increase in total β-casein after 8 weeks of supplementing the feed additive of *A. taxiformis*. The shift with the increase in caseins located within the casein micelle and a reduction in κ-casein, located at the outer layer of the micelle suggests a possible increase in micelle size (Walstra et al. 2005). Further studies are of interest to see if there is an effect of potential changes in micelle size and structure, which could affect dairy processes such as increased coagulation time, affecting the fermented dairy products and cheese. Feeding *A. taxiformis,* has also showed reduction in DMI, milk yield and fat concentration during feeding trials, (Muizelaar et al. 2021), due to unbalanced uptake of the algae, or refusal of intake, an effect that should be further investigated to avoid health issues of the ruminants and effect on productivity. The other plant derived additives are found naturally in the feed in varying degree. Similar for saponins, tannins, and essential oils , the scientific basis for their potential to mitigate enteric methane is today insufficient (Gerber et al. 2013). Altering organoleptic features of milk is an unwanted effect which has been reported (Silva et al. 2020), and long-term exposure is proposed to lead to an adaptation of the rumen microbiota, reducing the anti-methanogenic effect (Gerber et al. 2013).

Surveys of consumers' and dairy farmers' attitudes to methane reducing feed additives

The results from the surveys on attitudes to methane reducing feed additives showed optimism both among consumers and producers, although producers considered the impact of ruminants as much less prominent compared to the consumers, 78% and 31% respectively (figures 1 and 2). These differences also affected the thoughts on the need for methane reducing feed additives among the two groups. Consumers (60%) were more positive to the use of methane reducing feed additives compared to the farmers (48%). The opposition to the use of these additives was larger in the group of farmers (23%) compared to consumers (11%). A substantial proportion of the respondents in both groups were neither positive or negative or had no opinion in the matter. One reason brought up in the surveys was the lack of knowledge in the topic, which contributed to insecurities and disbelief to the additives. Information is thus an important task to have in mind when launching a new method of mitigating enteric methane. Increasing the knowledge amongst farmers and consumers by use of a good scientific basis could therefore encourage usage of the additives in practise. Milk is generally seen as a natural product and adding a foreign additive into the production chain could be perceived as negative. Eco-schemes offer a way of financing the introduction of the additives into practise if included in an EU-members CAP strategic plan. This is yet not the case in Sweden CAP strategic plan for 2023-2027. If it would be included it would give an opportunity to reduce the economic hinderance for the farmers and avoid higher costs for the milk produced for the consumers (European Commission 2021).

6. Conclusion

Methane reducing feed additives, e.g., 3-NOP or *A. taxiformis* have the capability of achieving a 30% reduction of enteric methane by targeting and inhibiting different steps in the methanogenesis, without impairing physiological functions, e.g., rumen fermentation and milk yield. Potential effects on milk composition resulting from the use of feed additives should be studied further. Effects on milk fat composition and milk protein profile have been observed, it is thus important to secure that there are no effects on product quality or processability. The active compound in *A. taxiformis*, i.e., $CHBr3$, and the residual product of 3-NOP, i.e., nitrite, are compounds which in larger quantities are associated with health concerns in humans and ruminants. Further studies examining factors affecting the excretion and accumulation of the compounds is an area of research needed to ensure a safe long-term use.

The optimism to the use of methane reducing feed additives amongst consumers, and the economic opportunities to fund an implementation of the additives encourage further studies on the effect on milk constituents and their functional properties.

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Popular science summary

Reducing climate impact by reducing greenhouse gas emissions is a global responsibility that all industries should strive to contribute to. The cows in agriculture are known to emit the greenhouse gas methane. Methane is formed when the cows' microbiota breaks down feed in the rumen. The function of this degradation process is to remove residual products from the feed degradation. The cows can eat difficult-to-digest types of feed such as grass, and at the same time extract nutrients from the feed that we humans cannot. Cows contribute a range of foods in the form of meat and dairy products and are important for maintaining open landscapes and increased biodiversity. Which provides important contributions to reduce climate impact. Can you then reduce those negative methane emissions while maintaining the good properties of the cows?

A new method approaching dairy production is to reduce methane emissions through methane reducing feed additives. These feed additives come in different forms and can reduce methane emissions by up to 30% without having any negative impact on cows' health or milk production. Two types of additives closest to introduction in dairy production are the chemical substance 3-nitrooxypropanol and the red alga *Asparagopsis taxiformis*. Methane production takes place through seven different steps in the cows' rumen and these additives block important components in the last steps that are necessary for the final formation of methane. An increased reduction of methane has led to a reduction in milk production and uptake of nutrients from the feed. Further studies in the subject should focus on seeing the long-term effects of the use of these additives to ensure product quality and ensure that the methane reduction effect is not reduced over time by the microbiota adapting to the additives.

The attitude towards the use of these methane reducing feed additives amongst both consumers and producers compiled through surveys showed optimism to the use of the additives. Which encourages further studies to ensure that no effects on the quality of dairy products occur.

Acknowledgements

I am forever grateful for my supervisors Åse Lundh, Maria Karlsson and Victoria Thuillier for the continuous support and cheers during my thesis writing process. A special thanks to Åse who helped structuring my writing process, your support really facilitated the writing. Many thanks to Maria and Victoria at Lantbrukarnas Riksförbund (LRF Dairy) who contributed with their expertise and knowledge in the field and provided me with surveys evaluated in this study which gave a holistic perspective to the thesis.

I would like to give a special thank you and gratitude to Rebecca Danielsson who gave me material from her own study to perform the laboratory part of the work. And a big thank you to Monika Johansson who gave me the opportunity and support to carry out the laboratory part, it was really appreciated.

To conclude, I want to thank my family and friends who are always there and support me no matter what. And an extra thank you to Emmy Nyberg for your great support and study company. Your company and our coffee made this study time much easier.

Appendix 1

The pH measurements of the milk samples collected week 0 and week 8 in each milk sample from the group of cows receiving 0,3% OM of *A. taxiformis* after control week 0.

MILK SAMPLE	W.0 pH	W.8 pH
1. 1967	6,58	6,71
2. 2011	6,64	6,62
3. 1931	6,70	6,71
4. 2117	6,77	6,68
5. 2165	6,77	6,68
6. 2163	6,85	6,82
7. 2171	6,65	6,61
8. 2120	6,80	6,64
9. 1823	6,79	6,78
10. 1986	6,77	7,10

Appendix 2

Description of the sample buffer and run buffer used in the protein profile laboratory in this study.

Sample buffer (SB)	**M**	**C (M)**	**g/0,05L**
Triss	121,14	0,167	
EDTA	372,24	0,067	
MOPS	209,26	0,042	
Urea*: *** 0,3L of 6M urea stock, with 0,05% MHEC (0,15g) + 5,4 g ion exchange resin mixed and stored overnight and filtered**	60,06	6,00	
MHEC		0,05%	
DTT** ***0,079g/10ml added to sample buffer before sample preparation**	154,25	0,017	
Run buffer (RB)	M	C (M)	g/0,2 L
Trisodium citrate	294,14	0,02	
Citric acid	210,14	0,19	
MHEC		0.05%	
Urea*	60,60	6,00	

Appendix 3

The questions asked in the consumer survey performed by NOVUS, and the question asked to dairy farmer panel of LRF Dairy, and answer used as the foundation for the resulting questions in the result section of the report.

- **The consumer survey questions:**
  - What is your general view on dairy cows' climate impact today?
  - Imagine that there was a Product that a dairy cow could eat and thereby greatly reduce her climate impact as methane emissions are reduced. The product does not affect the cow, the milk or the dairy products. What is your general attitude towards giving dairy cows a Product that reduces her climate impact?
  - Motivate your answer to the question about a product that reduces the cow's climate impact.
  - Do you think that your consumption of milk and dairy products would be affected if the milk came from cows that ate a product that reduces the cow's emissions of methane gas?

- **LRF Dairy Farmers panel questions:**
  - What is your general view on the climate impact of dairy crosses today?
  - Imagine that there was a product that dairy cows could eat and thereby greatly reduce the climate impact, as emissions of methane gas are reduced. The product does not affect the cow, the milk or the dairy products. What is your general attitude towards giving dairy cows a product that reduces her climate impact?
  - Feel free to motivate your answer to the question about a product that reduces the cow's climate impact.

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