# Effect of botanically diverse pastures on the milk fatty acid profiles in New Zealand dairy cows

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**Nyckelord/ *Key words*:** Dairy cow, milk fatty acids, CLA, diverse pastures, secondary plant metabolites

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# Abstract

Botanically diverse pastures are commonly used in New Zealand to reduce the ruminant environmental impact by reducing the methane production from the rumen digestion. In order to evaluate the effects diverse pasture species have on the milk fatty acid profile seventy-two lactating Friesian-Jersey crossbred dairy cows were used in a randomised block design with two replicates of six treatments. Six different pasture mixtures were fed at a daily allowance of approximately 15 kg DM/cow/day. The mixtures were categorised as either a simple or a diverse pasture. The simple mixtures all contained white clover with the addition of either a standard diploid perennial ryegrass (RG), a diploid high sugar ryegrass (HS) or tall fescue (TF). The three diverse mixtures included each of the simple mixtures with the addition of either chicory, plantain, prairie grass and red clover (RGD), chicory, plantain and big trefoil (HSD), or chicory, plantain, prairie grass and lucerne (TFD). Milk samples were collected and the fatty acid profile was analysed using Fatty Acids Methyl Esters (FAME) analysis. The proportion (g/100g of milk fat fatty acids) of linoleic and linolenic acid increased while the proportion of *cis*-9, *trans*-11 $C_{18:2}$ (CLA) decreased in milk from cows grazing the diverse pastures compared to cows fed the simple pastures. These changes were probably associated with a shift in the rumen microbial population or rumen metabolic routes caused by several secondary metabolites present in some plants, e.g. herbs and legumes. As a consequence the lipolysis and biohydrogenation decreased, resulting in an increased rumen outflow of linoleic and linolenic acid. These assumptions are based on the reported effects of secondary metabolites on rumen methanogenesis. The results of this study suggest that it is possible to change the milk fatty acid profile through inclusion of diverse plant species in the pastures which provides opportunities to change the fatty acid composition to become more beneficial from a human health perspective.

# Sammanfattning

Betesmarker bestående av varierande betesväxter används runt om i Nya Zeeland för att minska idisslarnas miljöpåverkan genom att minska metanproduktionen från vommen. För att utvärdera effekten dessa varierande betesväxter har på mjölkens fettsyrasammansättning användes 72 lakterande mjölkkor av en korsning av raserna nya zeeländsk Holstein-Friesian och Jersey i en studie med randomiserad block design med två repetitioner av sex behandlingar. I försöket användes sex olika blandningar av betesväxter och betestillgången var ungefär 15 kg TS/ko/dag. Blandningarna kategoriserades som antingen enkla eller mångfaldiga beten. De enkla betesblandningarna innehöll alla vitklöver med tillsatsen av antingen vanligt engelskt rajgräs (RG), engelsk rajgräs med hög sockerhalt (HS), eller rörsvingel (TF). De tre mångfaldiga blandningarna innehöll en av de enkla blandningarna med tillskottet av antingen cikoria, groblad, präriegräs och rödklöver (RGD), cikoria, groblad och stor käringtand (HSD), eller cikoria, groblad, präriegräs och lusern (TFD). Mjölkprover insamlades och mjölkens fettsyraprofil analyserades med hjälp av fettsyrametylesteranalys (FAME). Andelen (g/100g av totala fettsyror) linol- och linolensyra ökade medan andelen *cis*-9, *trans*-11 $C_{18:2}$ (CLA) minskade i mjölk från kor som gick på de mångfaldiga betesmarkerna. Förändringarna i mjölksammansättningen berodde antagligen på en förändring bland mikrobpopulationen eller i de metaboliska vägarna i vommen orsakat av sekundära metaboliter som finns i vissa växter, t.ex. baljväxter och örter. Som följd minskade lipolysen och biohydrogeneringen i vommen med ett ökat utflöde av linol- och linolensyra som följd. Dessa antaganden är baserade på de bevisade effekter som sekundära metaboliter i vissa växter har på vommens metanproduktion. De resultat som presenterats i den här studien indikerar att det är möjligt att ändra mjölkens fettsyraprofil genom att inkludera mer mångfaldiga växtarter i betesvallen. Detta ger möjligheter att förändra mjölkens fettsyrasammansättning så att den blir mer hälsosam för mänsklig konsumtion.

# Table of contents

# Introduction

New Zealand accounts for about 2.2% of the total milk production in the world which makes the country the world's eighth milk producer. Their dairy production is mainly pasture based since the milder winter climate makes it possible to keep the animals outdoors throughout the year with only some supplementary feeding during the winter months. In the last decades the dairy farmers have been aiming for an increased milk production and therefore focus has been on increasing the productivity and quality of the pastures. Simultaneously, the New Zealand dairy industry has been held to account for a big part of the country's environmental impact since the animals produce large amounts of methane per kg milk due to their forage based diets. In order to achieve this goal a lot of focus has been put on improving the quality and composition of the pastures. Research has shown that by altering the pasture composition both the milk production and methane production in the animal may be improved. Researchers have suggested that some plant species, such as birdsfoot trefoil (*Lotus corniculatus*), contain metabolites that may affect the rumen environment, resulting in a higher utilisation efficiency of the dietary nitrogen and lower methane production (Woodward *et al.,* 2004; Woodward *et al.,* 2009; Williams *et al.,* 2011).

Recently, inclusion of novel plant species into the pasture mixtures, such as chicory and plantain, has come to practise in order to reduce the environmental impact even further (Roca *et al.,* 2010). Inclusion of these diverse species provides an even greater opportunity to manipulate the fermentation patterns in the rumen and reduce the methane emissions due to their various nutritional and chemical compositions. Although these plant species are commonly used by farmers in New Zealand nowadays, their impact on the milk composition needs to be further investigated. One of the most variable components in milk, both in absolute amounts and in relative proportions, are the milk fat fatty acids. Due to the fact that their composition accounts for many of the milk's physical properties, increasing the fat content in milk has long been a major goal when feeding dairy cows. Some studies indicate that inclusion of botanically diverse plant species in the pastures increases the content of polyunsaturated fatty acids (PUFA) in ruminant products (Kraft *et al.,* 2003; Lourenço *et al.,* 2005). Increasing the content of PUFA in milk is currently of great interest as researchers have found that certain of these fatty acids have beneficial effects for human health and may act as anticarcinogenic agents (Parodi, 1997a; Parodi, 1999). Since the easiest way to affect the fatty acid profile in milk is by nutritional changes, including more diverse plant species in the pasture mixtures may lead to opportunities to increase these fatty acids in milk even further.

# Aim and hypothesis

The aim of this study was to examine how the milk fat fatty acid profiles in milk from lactating Friesian-Jersey crossbred dairy cows is affected when grazing botanically diverse pastures.

The hypothesis is that the milk fatty acid profiles will differ between cows with respect to whether they receive diverse pastures or not, especially in the proportion of long chain fatty acids which are of dietary origin. The proportion of certain PUFA, including linoleic acid, linolenic acid and *cis*-9, *trans*-11 $C_{18:2}$, will be higher in the milk of cows on the diverse pasture treatments.

# Literature review

## *Mixed pasture species for milk production*

New Zealand's pastoral farming for dairy cows is normally based mainly on the permanent mixture of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) (Powell *et al.*, 2007). The production and quality of these pastures is often limited during the summer months, partially due to a combination of hot weather and a shortage of rain. Botanically diverse pastures, however, have been shown to improve the yield of the pasture (Daly *et al.*, 1996), as well as enhance its nutritional profile (Sanderson *et al.*, 2003). Moreover, combining different plant species in the pasture provides an opportunity to maintain the productivity of the pastures in the summer months, as well as to manipulate the fermentation patterns in the rumen with possibilities to reduce ruminant livestock methane emissions (Roca *et al.*, 2010). The animals' utilisation of the pasture may also be increased which is of great importance for the farmers since dairy farming in New Zealand heavily relies on the pasture-based systems. Inclusion of other plant species may also increase milk production and alter milk composition and dietary CP content (Waugh *et al.*, 1998). Including a lot of legumes in the animal diet often result in increased milk production since legumes have a higher nutritive value than grasses (Ulyatt, 1981, cited by Cosgrove *et al.*, 2006). However, a major problem with mixed species pastures is that they often contain insufficient amount of clover to capture their high nutritional value as extra milk production (Cosgrove *et al.*, 2006) resulting in a lower increase in production than is possible. By combining species that interact with each other successfully the milk yield may still be increased without decreasing the species diversity in the pasture.

### High sugar ryegrasses

Increasing the content of water soluble carbohydrates (WSC) in ryegrass has long been of great importance in plant breeding and management in New Zealand since it is associated with a higher supply of readily-available energy for the degradation of plant protein in the rumen (Kingston-Smith & Theodorou, 2000). Thus, the conversion of plant protein into microbial protein is improved and more protein becomes available for absorption in the intestine. High sugar ryegrasses are promoted for their ability to increase milk yield, although the animal responses have been inconsistent. Miller *et al.* (2001b) demonstrated that milk yields from cows offered a high sugar ryegrass diet were higher, compared to a control group. Contrary to these results, Tavendale *et al.* (2006) found no difference in either milk production or milk fat concentration between cows fed pastures with different sugar content. However, coherently for these studies, the utilisation of dietary nitrogen (N) for milk production was improved for cows on a high sugar ryegrass diet (Miller *et al.*, 2001a; Miller *et al.*, 2001b; Tavendale *et al.*, 2006) and more N was excreted in the milk and less in urine (Moorby *et al.*, 2006). According to these diverse results there does not appear to be any unswerving relationships between the concentration of WSC and milk production (see also Edwards *et al.*, 2007). Cosgrove *et al.* (2009) investigated the chemical composition of three ryegrasses with different WSC content to establish the effect they had on animal performance. The results were inconsistent, although the authors speculated whether the reduction in both protein and fibre, at the expense of the higher sugar content, might enhance the effect WSC have on the nutritional value of ryegrasses. A higher sugar content could also affect the fatty acid profile in milk as it leads to a lower pH in the rumen which will affect the microbial population and the biohydrogenation process. Nevertheless, the various responses to WSC content in the diet indicate that there are other constituents in the plants that affect the animal performance, apart from the higher sugar content.

## Tall fescue in pastures

In New Zealand, dairy farming has expanded into dry areas, such as Canterbury, where irrigation is necessary to maintain the high production performances of the pastures. Since water is a limited source alternative grass species to ryegrasses that have higher water utilisation efficiency have been investigated. Minneé *et al.* (2010) investigated the establishment and production of pastures which either contained perennial ryegrass (*Lolium perenne*) or tall fescue (*Lolium arundinacea*). They observed that the perennial ryegrass pasture had a higher annual yield in the year of establishment, although the pasture containing tall fescue had higher yields of dry matter over the next years. Tall fescue also had higher water use efficiency in summer than ryegrasses. The authors concluded that tall fescue is a profitable alternative to ryegrass in dry regions in New Zealand. However, tall fescue has a slower establishment than ryegrasses and is more difficult to manage in a pasture since it is unable to compete with other grasses in a mixed sward (Easton *et al.,* 1994; Monk *et al.,* 2009). Nevertheless, tall fescue combined with chicory has shown production advantages compared to ryegrasses in soils with moderate moisture levels during the summer (Rollo *et al.,* 1998).

## Alternative pasture species

Recently, novel plant species have been introduced to the pasture mixtures in New Zealand, including chicory (*Cichorium intybus*), plantain (*Plantago lanceolata*) and big trefoil (*Lotus pedunculatus*). How these species will interfere with the rumen environment *in vivo* needs to be investigated. However, Roca *et al.* (2010) examined the methane production from chicory, plantain, birdsfoot trefoil (*Lotus corniculatus*) and ryegrass (*Lolium perenne*) when incubated *in vitro*. According to their results the methane production for chicory, plantain and birdsfoot trefoil was lower than for ryegrass throughout the study. They speculated whether the lower methane production for both chicory and plantain could be due in part to their high digestibility and ratio between water soluble carbohydrates and structural carbohydrates (WSC:SC). A high WSC:SC ratio is associated with increased passage rate through the rumen, due to rapid particle breakdown and low retention time in the rumen (Barry, 1998; Moss *et al.,* 2000). Roca *et al.* (2010) concluded that, due to their high WSC:SC ratio, legumes and herbs, such as chicory, plantain and birdsfoot trefoil, result in lower short-term *in vitro* production of methane, compared to ryegrass. Ramírez-Restrepo and Barry (2005) reached the conclusion that, in order to reduce the methane production and increase animal productivity, the most important plant characteristics are a high ratio of WSC:SC along with the presence of condensed tannins (CT) and possibly other secondary compounds. Condensed tannins are phenolic plant secondary compounds present in a number of legumes, including birdsfoot trefoil (*Lotus corniculatus*), big trefoil (*Lotus pedunculatus*) and in the flowers of white clover (*Trifolium repens*) (Terrill *et al.,* 1992). They act by binding to plant protein, forming complexes and making them unavailable for rumen degradation. As a result the amount of ammonia released from rumen digestion is reduced (Waghorn, 2008). Table 1 shows the presence of CT and other secondary compounds in some temperate forage species. Hence, these plant species may be used as feed stuffs for dairy cows in order to reduce the environmental impact.

*Table 1. Concentration of condensed tannins (CT) and other secondary compounds in temperate forage species. Adapted from Ramírez-Restrepo and Barry (2005)*

| ***Forage*** | ***Total CT content (g/kg DM)*** | ***Other secondary compounds*** |
|---|---|---|
| Perennial ryegrass<br>(*Lolium perenne*) | 1.8 | Endophyte alkaloids<br>12-30 mg/kg DM |
| Birdsfoot trefoil<br>(*Lotus corniculatus*) | 47 | 0 |
| Big trefoil<br>(*Lotus pedunculatus*) | 77 | 0 |
| White clover<br>(*Trifolium repens*) | 3.1 | Cyanogenic glycosides |
| Red clover<br>(*Trifolium pratense*) | 1.7 | Iso-flavones 7-14 g/kg DM |
| Lucerne<br>(*Medicago sativa*) | 0.5 | Coumestrol<br>0-100 mg/kg DM |
| Chicory<br>(*Chicorium intybus*) | 4.2 | Sesquiterpene lactones<br>3.6 g/kg DM |
| Plantain<br>(*Plantago lanceolata*) | 14 | Iridoid glycosides<br>Catapol 8 g/kg DM<br>Acubin 22g/kg DM |

Chicory has during the last decades gained popularity in New Zealand, most likely due to its advantageous growth patterns as a summer forage crop (Li *et al.*, 1997). Research has shown that chicory increases the fat content in milk, as well as the milk production, when offered as a supplement to pasture (Waugh *et al.,* 1998), possibly due to its high content of unsaturated fatty acids (Soder *et al.*, 2006). Its high digestibility of organic matter results in a rapid breakdown in the rumen and a high rumen fractional outflow rate (Barry, 1998). The high passage rate through the rumen is probably the reason that the feed intake often is higher in animals fed chicory, which most likely explains the increased milk production. Although chicory has a lower content of N than ryegrasses the duodenal flow of non-ammonia N is similar, possibly due to increased microbial production of proteins in the rumen which are transferred to the intestine.

Inclusion of plantain in the pasture mixtures has resulted in increased production and a more even distribution of dry matter yield throughout the year in the pasture (Moorhead & Piggot, 2009), probably due to its high drought tolerance and considerable summer heat tolerance (Stewart, 1996; Rumball *et al.*, 1997). Although few studies have investigated how the plant affects milk production in dairy cows, several studies have demonstrated positive influence on production in lambs and ewes, relative to perennial ryegrass (Moorhead *et al.*, 2002; Judson *et al.*, 2009; Hutton *et al.*, 2011). Furthermore, plantain contains antimicrobial compounds that appear capable of interfering with the rumen fermentation process and altering the volatile fatty acid composition which potentially can affect animal performance and milk composition (Deaker, 1994, cited by Stewart, 1996). Sanderson *et al.* (2003) investigated the nutritive value of both plantain and chicory from different harvests to determine their potential use as forage crops. They concluded that the herbs had a relatively high nutritive value, compared to other forage species, and that their inclusion in the pasture mixture could enhance its nutritional profile. Even though chicory and plantain have a high nutritional value their presence must be balanced against their lack of persistency in the sward in order to maintain a high pasture production and animal performance.

Although different plant species may affect the fatty acid composition in milk the main influencing factor is the amount and composition of dietary fat (Khanal & Olson, 2004). Thus the fatty acid composition in plants will now be discussed, followed by an introduction to the different fatty acids present in milk and how they are synthesised in the dairy cow.

## Fatty acids in plants

A traditional diet for dairy cows is composed of fresh or conserved forage and concentrate. However, these diets differ greatly among different regions in the world, as well as among seasons of the year. Therefore, the fatty acid composition in milk probably varies over the year and between different regions. In New Zealand, where the animal production systems are mostly pasture-based, altering the lipid profile in the pasture species is the easiest and most economical way to change the fatty acid profile in dairy milk. Today, the main goal of ryegrass selection in New Zealand is to increase the dry matter yield (Edwards *et al.*, 2007), while selecting for other traits, e.g. improving its nutritive value is relatively rare.

In fresh forage the total fat content comprises about 6-7% of the dry weight of leaf tissue, of which 40-50% are fatty acids (Harfoot & Hazlewood, 1997), present predominately in galactolipids and phospholipids (Bauman *et al.*, 1999). In pasture, the fatty acids are mainly unsaturated (average 70-90%), where linoleic acid and linolenic acid predominate (Harfoot & Hazlewood, 1997). Clapham *et al.* (2005) investigated the fatty acid composition in different plant species at three different harvest times (Table 2). They found that the fatty acid composition varied between the different harvests, where the trend was a declining content in most plants during their development.

*Table 2. Concentration (mg/g of DM) of certain fatty acids in different plants at three different harvest times with three weeks interval. Adapted from Clapham et al. (2005)*

| **Plant material** | **Harvest** | $C_{16:0}$ | $C_{16:1}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{18:3}$ |
|---|---|---|---|---|---|---|---|
| Perennial ryegrass | 1 | 6.99 | 0.94 | 0.30 | 1.46 | 6.76 | 34.7 |
| | 2 | 6.35 | 0.74 | 0.28 | 1.01 | 5.74 | 31.5 |
| | 3 | 5.91 | 0.56 | 0.32 | 0.71 | 5.47 | 26.8 |
| Tall fescue | 1 | 5.91 | 1.23 | 0.24 | 1.54 | 5.70 | 28.4 |
| | 2 | 4.94 | 0.97 | 0.22 | 1.03 | 4.12 | 25.3 |
| | 3 | 3.78 | 0.53 | 0.16 | 0.64 | 3.01 | 17.1 |
| White clover | 1 | 6.52 | 1.01 | 0.54 | 1.40 | 8.23 | 26.7 |
| | 2 | 5.62 | 0.75 | 0.47 | 0.89 | 5.89 | 20.3 |
| | 3 | 4.85 | 0.59 | 0.44 | 1.21 | 6.27 | 17.8 |
| Chicory (Puna) | 1 | 7.39 | 1.25 | 0.25 | 1.24 | 9.69 | 42.5 |
| | 2 | 5.65 | 0.81 | 0.22 | 0.43 | 7.17 | 24.2 |
| | 3 | 5.01 | 0.63 | 0.22 | 0.31 | 5.88 | 19.8 |
| Plantain | 1 | 6.64 | 1.03 | 0.37 | 0.73 | 8.68 | 26.1 |
| | 2 | 5.28 | 0.67 | 0.38 | 0.39 | 6.19 | 22.6 |
| | 3 | 3.72 | 0.39 | 0.34 | 0.30 | 4.49 | 15.2 |

## Factors affecting the fatty acid profile in plants

The fatty acid profile in plant species are constantly subject to turnover and lipases are always present in the living plant, degrading the lipids. This will, however, not have an important influence on the fatty acid composition during normal growing conditions. Nevertheless, the fatty acid profile may change significantly during senescence of the plant, after detachment and during storage (Elgersma *et al.,* 2003b). Environmental factors have a huge influence on the fatty acid composition in plants as well, such as temperature, light intensity (Hawke, 1973; Dewhurst & King, 1998), stage of maturity and season (Elgersma *et al.*, 2003b). The leaf/stem ratio may also have an effect on the fatty acid composition in early season due to the fact that the galactolipids are mainly located in the metabolically active leaves (Van Soest, 1994). Furthermore, nitrogen application increases the concentration of fatty acids in plants but do not have any impact on their profile (Elgersma *et al.,* 2005).

## Fatty acid composition in fresh grass and silage

At harvest, the concentration of linoleic and linolenic acid decreases in forage due to oxidation and degradation by different enzymes, a plant defence mechanism initiated in damaged tissues (Elgersma *et al.,* 2003b; Dewhurst *et al.,* 2006). Thus, there is some risk that the content of fatty acids will be lower in the fermented silage, depending on the ensiling method used. Elgersma *et al.* (2003a) examined the fatty acid composition in fresh grass versus ensiled forage. According to their results 98% of the fat in fresh grass was present as esterified fatty acids (EFA) whereas, in ensiled grass, 27-73% of the total fatty acids consisted of free fatty acids (FFA). Thus, during the conservation process important PUFA that are precursors for fatty acids in milk may be lost. Dewhurst and King (1998) found that the content of total fatty acids was reduced by 30% when the grass was wilted prior to ensiling and the concentration of linolenic acid was reduced by up to 40%. Nevertheless, ensiling may only have a minor influence on the fatty acid concentration, provided compaction and sealing of the silos are effective (Doreau & Poncet, 2000). Additives, such as formic acid or formalin, also reduce losses significantly.

Agenäs *et al.* (2002) examined how the composition of different milk fatty acids changed when cows where turned out to pasture after being kept indoors for the winter period. After turnout there was an increase in the long chain fatty acids, which originate from dietary fat or adipose tissue. Simultaneously there was a reciprocal decrease in the *de novo* fatty acids, synthesised within the mammary gland. The results indicate that the shift in fatty acid composition in milk fat was caused by a higher proportion of unsaturated fatty acids from pasture which consists of more long chain fatty acids, especially linolenic acid which is the main precursor for *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 $C_{18:2}$ in milk (Agenäs *et al.,* 2002; Elgersma *et al.*, 2004). Similar results were shown by Kelly *et al.* (1998b). In their study two groups of Holstein cows were divided into either a control group fed a total mixed diet, or a grazing group which was gradually adjusted to a diet consisting of fresh pasture. Milk samples were analysed and the result showed that cows on pasture had higher milk fat concentrations of CLA than cows fed a total mixed diet, which has been confirmed in later studies (White *et al.,* 2001; Kraft *et al.,* 2003; Elgersma *et al.,* 2004). Furthermore, grazing cows had a lower concentration of short and medium chain fatty acids in their milk, due to decreased milk fat synthesis in the mammary gland.

## *Fatty acids in milk*

Milk fat is composed by a large number of fatty acids of varying length and saturation, most of which are present esterified to glycerol as triacylglycerides. The exact number of fatty acids is still unclear but has been estimated to be over 400 (Bauman & Griinari, 2003). However, a large proportion of these acids are only present as trace amounts in the milk, while about 15-20 fatty acids represent approximately 90% of the total number (Bauman *et al.,* 2006). The fatty acids are divided into two different groups, according to the length of their carbon chains. Approximately 50-70% of the fatty acids are composed of long carbon chains with 16 carbons or more, while the other 30-50% have carbon chains that consist of 14 carbons or less, i.e. short and medium chain fatty acids (Santos, 2002). The different fatty acids in milk arise almost equally from the uptake of circulating, preformed fatty acids or from the synthesis of new fatty acids that occur in the mammary gland, i.e. *de novo* synthesis (Bauman *et al.,* 2006).

The typical composition of milk fat in dairy cows comprises of approximately 70% saturated fatty acids, 25% monounsaturated fatty acids and 5% polyunsaturated fatty acids (Grummer, 1990; Bauman *et al.,* 2006; Elgersma *et al.,* 2006). The polyunsaturated fatty acids represent approximately 2.3% by weight of the total fatty acids (Lindmark Månsson, 2003). The main ones are linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) which account for 1.6 and 0.7% by weight of the total fatty acids, respectively. The composition of fatty acids in milk can be affected by many different factors, such as genetics, stage of lactation, mastitis and ruminal fermentation (Palmquist *et al.,* 1993). The composition can also be affected by feed-related factors, i.e. it can be related to intake of fibre and energy, as well as by seasonal and local effects which may influence the feed composition.

Milk fat also consists of *trans* fatty acids, which represent approximately 3.2-5.2% of the total fatty acids (Aro *et al.,* 1998). The main *trans* fatty acids in milk are *trans*-11 $C_{18:1}$ (vaccenic acid) and different isomers of conjugated linoleic acid, of which *cis*-9, *trans*-11 $C_{18:2}$ (rumenic acid) represents more than 80% (Chin *et al.,* 1994). *Trans* fatty acids have long been considered to be less favourable from a human health perspective and have been associated with an increased risk of several chronic diseases, including cardiovascular disease and type-II diabetes (Willett, 2006; Erkkilä *et al.,* 2008). Nevertheless, scientists have during the last quarter decades found that some of these *trans* fatty acids have beneficial effects on human health and may act as functional food components in the milk (Bauman *et al.,* 2006).

### Bioactive components and CLA in milk

As a result of consumers' increased awareness of the association between diet and health much research has focused on to clarify the role specific food components have on health maintenance and disease prevention. Scientists have found that milk contains numerous components that have bioactive properties beneficial to human health, including several fatty acids, as shown in Table 3.

*Table 3. Milk fat components that have beneficial human health implications. Adapted from Bauman et al. (2006)*

| *Cancer* | *Cardiovascular Health* | *Immune Response* | **Bone Health** |
|---|---|---|---|
| CLA<br>Vaccenic acid<br>Sphingolipids<br>Butyric acid<br>13-methyltetradecanoic acid<br>Ether lipids | CLA<br>Stearic acid<br>Omega-3 fatty acids | CLA | CLA |

Among the different fatty acids there are primarily isomers of conjugated linoleic acid (CLA) that are of interest and about 75% of our daily intake of CLA comes from dairy products (Bauman *et al.*, 2006). The term CLA consists of 28 different isomers of octadecadienoic acid, where every positional isomer has four possible geometric pairs of isomers, i.e. *cis,trans; trans,cis; trans,trans* and *cis,cis* (Collomb *et al.*, 2006). Although 14 different forms of CLA occur naturally in milk fat (Lock & Bauman, 2004), it is the *cis*-9, *trans*-11 $C_{18:2}$ (Figure 1), which represents more than 80% of the total CLA in milk fat, that is of interest as a functional food component (Parodi, 1977; Aro *et al.*, 1998). *Cis*-9, *trans*-11 $C_{18:2}$ acts anticarcinogenic by inhibiting the growth of a number of cancer cell lines and by suppressing tumour development in the body (Parodi, 1997b). Other beneficial effects on human health are antiatherogenic effects, reducing the development of atherosclerotic lesions, and reducing total plasma cholesterol and low-density lipoproteins cholesterol concentrations. The presence of *cis*-9, *trans*-11 $C_{18:2}$ are related to the ruminal biohydrogenation of polyunsaturated fatty acids (PUFA) and it is an intermediate in the biohydrogenation of linoleic acid. However, the principal source of *cis*-9, *trans*-11 $C_{18:2}$ in milk fat are derived from endogenous synthesis in the mammary gland, which will be described later.

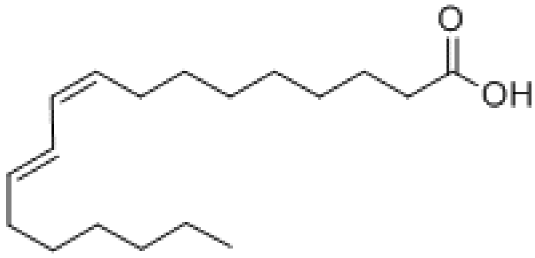

*Figure 1. Chemical structure of cis-9, trans-11* $C_{18:2}$.

## *Milk fat synthesis*

The synthesis of milk fat in dairy cows occurs from two different sources. The first source is uptake from circulation of dietary fat or adipose tissue which has been biohydrogenated in the rumen (Bauman *et al.*, 1999; Griinari & Bauman, 1999; Lock & Bauman, 2004). The fatty acids that are taken up from circulation are derived mainly from the intestinal absorption of dietary and microbial fatty acids, while lipolysis and mobilisation of body fat only accounts for less than 10% of the fatty acids in milk (Bauman & Griinari, 2003). The second source of milk fatty acids is from endogenous synthesis within the mammary gland, i.e. *de novo* synthesis. Short chain fatty acids (4 to 8 carbons) and medium chain fatty acids (10 to 14 carbons) arise almost exclusively from synthesis within the mammary gland while long chain fatty acids (>16 carbons) are derived from the uptake of circulating lipids (Elgersma *et al.*, 2003b). Fatty acids of 16 carbons in length originate from both sources. Since very little of the desired long chain fatty acids in milk ($C_{18}$) are synthesised by the cow itself these have to be ingested with the feed. The amount of dietary fat that is directly transformed into milk fat depends mainly on three different factors; the biohydrogenation in the rumen, the absorption and digestibility of the ingested nutrients, and the deposition of adipose tissue (Palmquist *et al.,* 1993). To understand how the fatty acid composition is influenced by nutrition, knowledge about the metabolic pathways of the synthesis of milk fat is of great importance.

## Fatty acid metabolism in the rumen

The fatty acids that originate from the ingested feed undergo two transformations in the rumen. Firstly the microbial lipases hydrolyse the ester linkages and secondly the unsaturated fatty acids are biohydrogenated by the rumen bacteria (Harfoot & Hazlewood, 1997; Bauman *et al.,* 1999). The bacteria can be divided into two different groups, A and B, based partly on their end products of biohydrogenation. Members of group A bacteria mostly hydrogenate linoleic acid and linolenic acid which result in the end product *trans*-11 $C_{18:1}$. Group B bacteria are capable of hydrogenating this *trans*-11 $C_{18:1}$ fatty acid into stearic acid. To which extent the fatty acids are hydrogenated depends on the type of diet, the degree of unsaturation of the fatty acids, and on the level and frequency of feeding (Jenkins, 1993).

The initial step in the biohydrogenation process of linoleic acid is isomerisation of the *cis*-12 double bond which leads to the formation of *cis*-9, *trans*-11 $C_{18:2}$ as the double bond is transferred to carbon-11 position (Harfoot & Hazlewood, 1997; Bauman *et al.,* 1999; Khanal & Dhiman, 2004). This is followed by a rapid hydrogenation of the *cis*-double bond of *cis*-9, *trans*-11 $C_{18:2}$ which is then converted into *trans*-11 $C_{18:1}$. However, the hydrogenation of the *trans*-11 $C_{18:1}$ to stearic acid occurs less rapidly and thus it accumulates in the rumen and may therefore escape to the intestines where it can be absorbed (Griinari & Bauman, 1999; Bauman & Griinari, 2001). An alternative route for the biohydrogenation of linoleic acid involves the formation of *trans*-10, *cis*-12 $C_{18:2}$, typical for diets that cause milk fat depression (Bauman & Griinari, 2001).

Linolenic acid is isomerised at *cis*-12 position, forming *cis*-9, *trans*-11, *cis*-15 $C_{18:3}$ (Harfoot & Hazlewood, 1997; Khanal & Dhiman, 2004). This fatty acid is then reduced at the *cis*-bonds and forms *trans*-11 $C_{18:1}$. The final step in the biohydrogenation of linolenic acid is similar that of linoleic acid. Approximately 75-90% of linoleic acid and 85-100% of linolenic acid are biohydrogenated in the rumen (Harfoot & Hazlewood, 1997). A schematic picture of the biohydrogenation process of both linoleic and linolenic acid is shown in Figure 2.

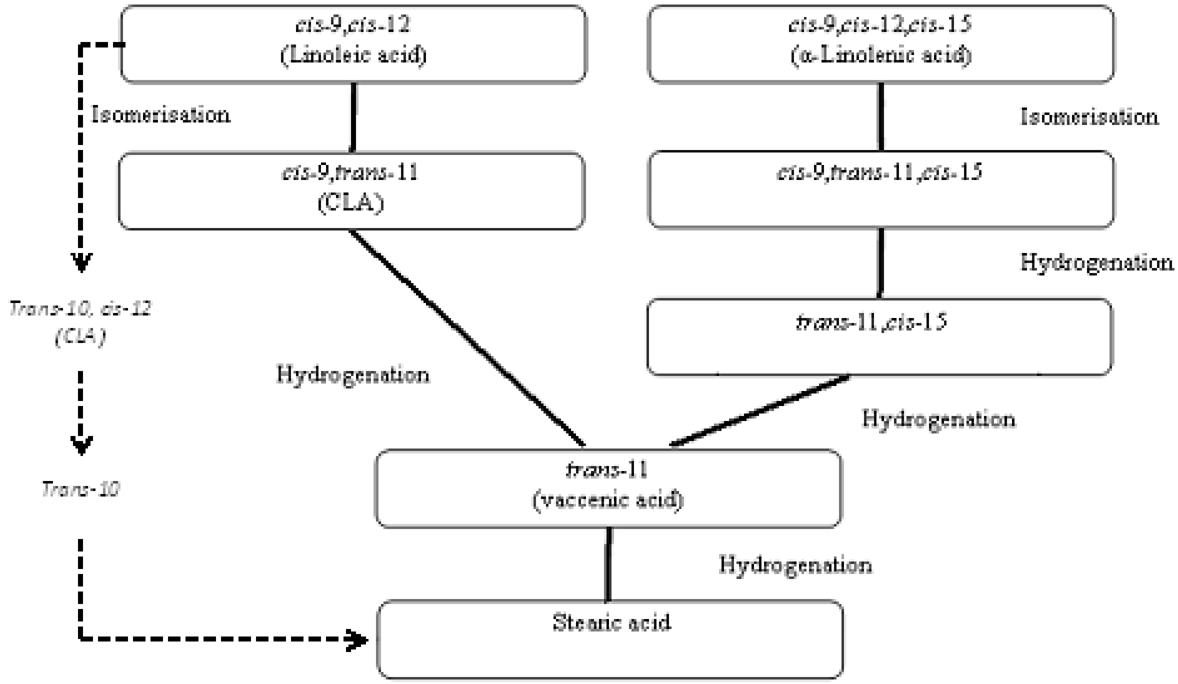

*Figure 2. Scheme for the biohydrogenation of linoleic acid and α-linolenic acid. Adapted from Griinari et al. (1999).*

As a consequence of the ruminal biohydrogenation the fatty acids that reach the small intestine are mainly saturated, although some intermediates from the biohydrogenation can escape the rumen. The two major ones are *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 $C_{18:2}$ (Lock & Bauman, 2004). From the small intestine the fatty acids are absorbed through the lumen wall and transported as triglycerides in VLDL (very-low-density lipoprotein) through the bloodstream to the udder. The uptake by the mammary gland depends on the action of LPL (lipoprotein lipase) which is located in the capillary walls. The fatty acids that have been mobilised from adipose tissue are transported as non-esterified fatty acids and their uptake by the mammary gland is proportional to plasma concentrations.

## Endogenous synthesis of CLA

As mentioned previously, *cis*-9, *trans*-11 $C_{18:2}$ is formed as an intermediate in the ruminal biohydrogenation and is rapidly hydrogenated into *trans*-11 $C_{18:1}$. However, *trans*-11 $C_{18:1}$ can be converted into *cis*-9, *trans*-11 $C_{18:2}$ in the mammary gland and in endogenous tissues by the action of the enzyme $\Delta^9$-desaturase (Griinari & Bauman, 1999; Griinari *et al.*, 2000). *Trans*-11 $C_{18:1}$ and *cis*-9,*trans*-11 $C_{18:2}$ are present in the milk at a ratio of 1:3 (Lock & Bauman, 2004) and about 70-80% of the *cis*-9,*trans*-11 $C_{18:2}$ found in cow milk originates from endogenous conversion of *trans*-11 $C_{18:1}$ (Griinari *et al.*, 2000; Lock & Garnsworthy, 2002). Both *cis*-9, *trans*-11 $C_{18:2}$ and *trans*-11 $C_{18:1}$ found in milk have been shown to have anticarcinogenic activity (Table 3), the latter because it can be converted to *cis*-9, *trans*-11 $C_{18:2}$ through the *de novo* synthesis in the mammary gland. Ingested *trans*-11 $C_{18:1}$ can also be converted into *cis*-9, *trans*-11 $C_{18:2}$ endogenously in mammals (Salminen *et al.*, 1998) and the conversion rate have been estimated to approximately 20% (Turpeinen *et al.,* 2002).

## *Increasing CLA content in milk fat*

The content of the CLA *cis*-9, *trans*-11 $C_{18:2}$ in milk fat is dependent on a number of different factors and can be increased several-fold, where the diet of the dairy cow is the most significant factor (Bauman *et al.,* 1999; Bauman & Griinari, 2003). The amount in the milk can be increased either by increasing the intake of its precursors in the diet, by reducing the level of biohydrogenation in the rumen, or by increasing the activity of the enzyme $\Delta^9$-desaturase, which converts *trans*-11 $C_{18:2}$ into *cis*-9, *trans*-11 $C_{18:2}$ in the mammary gland (Elgersma *et al.,* 2003b). The enzyme activity have, however, been proposed to be rather unaffected by the diet (Lourenço *et al.,* 2005b). According to Griinari *et al.,* (2000), the main focus should lie on increasing the ruminal formation of *trans*-11 $C_{18:1}$, rather than the production of *cis*-9, *trans*-11 $C_{18:2}$ itself. Therefore the most feasible alternative would be to feed a diet rich in *trans*-11 $C_{18:1}$ or to increase the formation of this fatty acid by the rumen biohydrogenation and its uptake in the duodenum. This statement was later supported by Vasta and Luciano (2011).

The most common methods to increase the content of *cis*-9, *trans*-11 $C_{18:2}$ are by feeding fresh pastures, or by addition of plant and marine oils to the diet (Dhiman *et al.,* 1999; Bauman & Griinari, 2003). The composition of the dietary fatty acids, along with other dietary factors, will affect the ruminal production of both *cis*-9, *trans*-11 $C_{18:2}$ and *trans*-11 $C_{18:1}$ (Bauman *et al.,* 1999). These factors involve both provision of lipid substrate for the production of *cis*-9, *trans*-11 $C_{18:2}$ or *trans*-11 $C_{18:1}$, as well as factors that affect the bacteria involved in the biohydrogenation process, i.e. changes in the rumen environment. Furthermore, diets rich in linoleic acid, as well as pasture based diets, have been shown to inhibit the hydrogenation of *trans*-11 $C_{18:1}$, resulting in this fatty acid being the main end product instead of stearic acid (Harfoot *et al.,* 1973; Harfoot & Hazlewood, 1997). Thus, *trans*-11 $C_{18:1}$ accumulates to a higher extent in the rumen and therefore more substrate can be absorbed and be available for endogenous synthesis of *cis*-9, *trans*-11 $C_{18:2}$. The milk content of *cis*-9, *trans*-11 $C_{18:2}$ may also be affected by the stage of maturity of the forage. Forage at the early growth stage gives an increase of *cis*-9, *trans*-11 $C_{18:2}$ in milk fat, compared to second-cut or late-growth forage (Chouinard *et al.,* 1998, cited by Bauman *et al.,* 1999). It is possible that the high amount of degradable carbohydrates in early grazed pastures may affect the rumen environment so that less *cis*-9, *trans*-11 $C_{18:2}$ and *trans*-11 $C_{18:1}$ is used by the rumen and therefore available for postruminal absorption (Shroeder *et al.,* 2004).

Another way to increase the content of *cis*-9, *trans*-11 $C_{18:2}$ in the milk is to increase the concentration of linoleic and/or linolenic acids in the diet. Lock and Garnsworthy (2002) examined how different diets affected the content of CLA in cows' milk. They discovered that there were only diets high in linoleic acid that resulted in increased production of *cis*-9, *trans*-11 $C_{18:2}$ in the rumen as well as in the milk. In another study (Dhiman *et al.,* 2000), a similar increase of *cis*-9, *trans*-11 $C_{18:2}$ for diets rich in linolenic acids has been found as well. In a study by Kelly *et al.* (1998a), groups of equally producing Holstein cows were randomly assigned to three different diets to which peanut oil (high oleic acid), sunflower oil (high linoleic acid) or linseed oil (high linolenic acid) where added. Milk samples were collected and analysed from all cows at the end of the treatment period. The results showed that the milk fat concentration of *cis*-9, *trans*-11 $C_{18:2}$ during the sunflower oil treatment differed significantly from the other treatments and was approximately 500% higher than for cows consuming traditional diets. Similar results have been observed when feeding diets rich in corn oil, which contains about 50% linoleic acid (McGuire *et al.,* 1996). Furthermore, diet supplementation of *cis*-9, *trans*-11 $C_{18:2}$ has been shown to lead to increased concentration of this fatty acid in milk fat (Chouinard *et al.,* 1998, cited by Bauman *et al.,* 1999). However, feeding diets high in plant oils often result in milk fat depression and may also decrease the milk protein percentage and yield (Bauman & Griinari, 2003).

## *Effect of feeding botanically diverse forages on milk fat synthesis*

Feeding ruminant animals diets consisting of more botanically diverse forages have resulted in changes in the milk fatty acid profile, making the milk healthier for human consumption (Lourenço *et al.*, 2008). Several studies have shown that diverse forage diets result in a higher proportion of PUFA in the milk, including *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 $C_{18:2}$, compared to feeding ryegrass-based diets (Kraft *et al.,* 2003; Lourenço *et al.*, 2005). In addition, the milk also has a higher content of linolenic acid even though the intake of this fatty acid does not differ significantly between diets (Table 4) (Lourenço *et al.*, 2005; Lourenço *et al.*, 2008; Petersen *et al.,* 2011).

*Table 4. Milk fatty acid profile, recovery of dietary linoleic ($C_{18:2}$ n-6) and linolenic ($C_{18:3}$ n-3) acid in the milk and intake of dairy cattle fed a less diverse forage (>600 g/kg DM of grasses) or a botanically diverse forage (>300 g/kg of herbs). Adapted from Lourenço et al. (2008)*

| ***Fatty acids*** | ***Control*** | ***Botanically diverse*** | ***P*** |
|---|---|---|---|
| *Fatty acids (g/100 g)* | | | |
| $C_{18:1}$ *t*10 + *t*11 | 1.99 | 2.84 | n.s. |
| $C_{18:2}$ n-6 | 1.07 | 1.22 | n.s. |
| CLA *c*9*t*11 | 0.866 | 1.43 | n.s. |
| $C_{18:3}$ n-3 | 0.788 | 1.06 | <0.05 |
| | | | |
| *Intake (g/d)* | | | |
| $C_{18:2}$ n-6 | 65.4 | 64.0 | n.s. |
| $C_{18:3}$ n-3 | 147 | 116 | n.s. |
| | | | |
| *Recovery (g/100g)* | | | |
| $C_{18:2}$ n-6 | 8.40 | 9.39 | n.s. |
| $C_{18:3}$ n-3 | 4.34 | 5.80 | <0.1 |

Petersen *et al.* (2011) studied the effects a herbage rich diet had on milk composition compared to a clover rich and a ryegrass rich diet. The herbage diet contained high levels of chicory, English plantain and salad burnet and resulted in a twofold increase in the transfer efficiency for linolenic acid. Furthermore, the herbage diet increased the transfer efficiency for linoleic acid by 28%, even though the diet did not contain higher levels of any of these fatty acids.

The increases of certain PUFA in milk from cows fed botanically diverse diets most likely result from an increased rumen outflow of *trans*-11 $C_{18:1}$ and linolenic acid to the intestine caused by a change in the fermentation patterns in the rumen (Lourenço *et al.,* 2005b). Correlations have been found between biohydrogenation patterns and the content of PUFA in plant species (Collomb *et al.,* 2002b) as well as between diverse plant species and the microbial activity in the rumen (Lourenço *et al.,* 2007a; Lourenço *et al.,* 2007b). Feeding botanically diverse forages to ruminants are associated with changes in the microbial populations in the rumen which are suggested to partially inhibit the hydrogenation of linoleic and linolenic acid. Consequently the conversion of *trans*-11 $C_{18:1}$ into stearic acid ($C_{18:0}$) becomes more sensible to inhibition, resulting in an accumulation of *trans*-11 $C_{18:1}$ in the rumen (Lourenço *et al.,* 2005b). The additional quantity of *trans*-11 $C_{18:1}$ can more easily escape the rumen and thus be available for absorption in the intestine. As mentioned earlier, *trans*-11 $C_{18:1}$ can be transformed into *cis*-9, *trans*-11 $C_{18:2}$ in the mammary gland and in endogenous tissues (Griinari & Bauman, 1999; Griinari *et al.*, 2000). Therefore this fatty acid is increased in the animal products as well. Lourenço *et al.* (2008) compiled results from several studies examining the effects of botanically diverse forages. They could not distinguish any correlation between the proportion of diverse forages in the diet and the proportions of *cis*-9, *trans*-11 $C_{18:2}$ in milk. Nevertheless, they suggested that the number of plant species in the diet could be associated with the changes in milk proportions of *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 $C_{18:2}$.

The inhibition of complete biohydrogenation in the rumen is most likely caused by a shift in the microbial population. It is possible that some herbs contain secondary plant metabolites that have antimicrobial activity and can therefore potentially modify the rumen biohydrogenation by inhibiting or promoting different microbial populations (Lourenço *et al.*, 2007b). These suggestions are based on the demonstrated effects some secondary plant metabolites, e.g. condensed tannins, have on rumen methanogenesis (Waghorn, 2008). Inclusion of CT in the diet has also been reported to increase the milk production and change the milk composition in dairy cows. Woodward *et al.* (1999) examined to which proportion the increase in milk yield and milk composition was due to the CT and which proportion was due to factors associated with the legumes themselves, e.g. increased herbage intake and improved forage quality. Their results concluded that CT contributed to approximately 42% of the increased milk production from a diet consisting of birdsfoot trefoil (*Lotus corniculatus*), compared to a ryegrass diet. However, they found that CT themselves had no significant effect on milk fat concentration, indicating that the higher concentrations of fatty acids in milk from cows fed the legume diet were a result of the legumes' higher nutritional value compared to ryegrass. The same results were later confirmed by Turner *et al.* (2005) and by Benchaar and Chouinard (2009). Although some research show that CT and other secondary plant metabolites have a beneficial impact on milk composition lack of direct evidence implies that further research within the area is essential.

Before continuing with the next part of this report it should be mentioned that most studies included in this review has compared different grass forages and silages of varying composition but not pasture diets. The effect of feeding botanically diverse forages on milk composition should be rather similar between silage and pasture diets but the amount and concentration of the milk solids might not be comparable with each other.

# Material and methods

## Experimental design

The experiment was conducted at Lincoln University Research Dairy farm during summer 2011 (27 January – 5 February). All sampling was carried out in compliance with Animal Ethics regulations.

A total of seventy-two lactating Friesian-Jersey crossbred dairy cows of different lactation numbers were used. After being blocked according to milk yield, live weight, body condition score, days in milk and age, cows were divided into 12 groups (6 cows/group) and randomly assigned to six replicated pasture treatments. The experimental design was randomised block design where two replicates of six treatments (Figure 3). Pasture allowance was, approximately, 15 kg DM/cow/day. The six different pasture mixtures used were categorised as either a simple or a diverse pasture. The three simple mixtures all included white clover (cv. Kopu II) and were sown with either a standard diploid perennial ryegrass (RG cv. One50), a diploid high sugar ryegrass (HS cv. Abermagic) or with tall fescue (TF cv. Advance). The three diverse mixtures included each of the simple mixtures with the addition of either chicory, plantain, prairie grass and red clover (RGD), chicory, plantain and big trefoil (HSD), or chicory, plantain, prairie grass and lucerne (TFD). In all six combinations the base grasses were infected with either AR1 or MaxP endophyte.

| | | |
|---|---|---|
| HS | RGD | TFD |
| HSD | TFD | TF |

| | | |
|---|---|---|
| TF | RG | HSD |
| RG | RGD | HS |

*Figure 3. The experimental design and pasture treatments.*

The experiment lasted for 8 days with a 4 day adaptation period followed by a 4 day sampling period. Animals were milked twice daily at 7am and 3pm, and received a fresh pasture allocation following the 3pm milking.

## Pasture measurements

Dry matter intake (DMI) was determined from random quadrate cuts which were harvested from each of the pastures pre and post grazing each day. The herbage was dried to a constant weight and dry weights were recorded. The calculations were made as following:

$$\mathrm{DMI} = \frac{[\text{Pre mass (kg DM ha}^{-1} - \text{post mass (kg DM ha}^{-1}] \cdot \text{area (ha)}}{\text{No. of animals}}$$

Samples from each replicate were thoroughly mixed prior to sub sampling. Two sub samples were made; the first one was immediately frozen for later analysis while the second sub sample was used to determine the botanical composition of the herbage. The fresh weight was recorded and the plant materials were sorted into either vegetative or reproductive ryegrass, different plant species, dead material or weeds. Each plant component was then dried separately at 60°C for 48h and dry weights were recorded. Based on these values the DM content of the whole diet was calculated. The chemical composition and digestibility was analysed by using a near-infrared spectrophotometer (NIRS. Model: FOSS NIRSystems 5000). The analyses for each of the pasture treatments and the inclusion level of the diverse plant species in the different pasture mixtures are presented in Table 5 and 6, respectively. The HSD pasture contained big trefoil but the inclusion level was too low to be measured, and values for big trefoil are not presented.

*Table 5. Chemical composition and calculated nutrient content of the different pastures. Values are presented as % of DM unless specified otherwise*

| | ***HS*** | ***HSD*** | ***RG*** | ***RGD*** | ***TF*** | ***TFD*** |
|---|---|---|---|---|---|---|
| DM *(% fresh forage)* | 22.68 | 16.93 | 23.54 | 16.73 | 18.25 | 16.90 |
| CP | 16.21 | 13.67 | 13.41 | 16.24 | 19.44 | 13.77 |
| WSC | 17.65 | 15.66 | 17.32 | 12.88 | 13.22 | 11.45 |
| NDF | 35.09 | 28.82 | 40.42 | 30.78 | 35.07 | 34.63 |
| ADF | 18.24 | 19.11 | 21.65 | 22.03 | 19.43 | 23.94 |
| Ash | 9.35 | 11.01 | 10.18 | 10.29 | 10.45 | 10.63 |
| OMD | 89.79 | 87.87 | 85.37 | 80.06 | 86.29 | 79.01 |
| ME *(MJ/kg DM)* | 12.53 | 11.95 | 11.90 | 11.26 | 11.94 | 11.00 |

*Table 6. Inclusion level (in % of DM) of different plant species in each of the pasture mixtures, along with the proportion of dead material*

| | ***HS*** | ***HSD*** | ***RG*** | ***RGD*** | ***TF*** | ***TFD*** |
|---|---|---|---|---|---|---|
| High sugar ryegrass | 75.35 | 22.63 | | | | |
| Standard ryegrass | | | 77.29 | 30.86 | | |
| Tall fescue | | | | | 70.94 | 2.73 |
| White clover | 1.91 | 0.85 | 2.20 | 3.90 | 23.79 | 4.97 |
| Chicory | | 56.69 | | 32.93 | | 53.08 |
| Plantain | | 3.91 | | 5.31 | | 8.74 |
| Lucerne | | | | | | 8.02 |
| Red clover | | | | 20.35 | | |
| Prairie grass | | | | 2.04 | | 9.95 |
| Dead plant material | 22.60 | 13.92 | 20.44 | 4.49 | 5.14 | 5.15 |

## *Sampling and analysis*

Milk samples were collected from both am and pm milkings on day 6 during the four day sampling period and milk yield was recorded (DeLaval Alpro Herd Management system, Hamilton, New Zealand). Sub samples were attained for composition of fat, protein, lactose and urea-N. The sub samples were frozen at -20°C until milk fatty acid methyl esters (FAME) analysis. To get a representative sample for each day the milk from the am and pm milkings were sub pooled for each cow based on their milk volume for that day. Before sub pooling, the samples were thawed to 37°C to get homogenized. FAME GC analysis (GC-2010, Shimadzu) was performed and peaks were identified by comparison of retention times with reference standards (Larodan, Sweden).

Cow number 3 and 13 were not sampled on the am milking and their samples were not analysed for FAME analysis.

### *Statistical analysis*

The data were analysed in two different ways; to examine the effect diverse pastures had on milk yield and milk fatty acid composition compared to simple pastures, and to examine the effect each diverse pasture mixture had compared to its simple mixture (HS vs. HSD etc.). In addition, milk yield data was analysed, and treatment means differences were tested, across all different pasture types.

In order to compare all the diverse mixtures to the simple mixtures a two-way ANOVA was used (GenStat v.14). The animals were grouped according to whether the treatment was a simple or a diverse mixture of each base grass (i.e. HS, RG, and TF) and the calculations were based on the mean values of each group for each of the fatty acids of interest. Treatments were included as fixed effects.

To investigate the effect each diverse mixture had when compared to its simple version a one-way ANOVA was used (GenStat v.14). Animals were grouped according to treatment (e.g. simple/diverse) and treatments were included as fixed effects.

The level of significance was set at 5% ($P < 0.05$) and levels of $P < 0.1$ were considered as tendencies.

# Results

## *Milk production*

The milk yields registered on the day of sampling are presented in Table 7. The milk yield did not differ significantly between cows on diverse compared to simple pastures. However, cows on pasture treatment TF had a significantly ($P = 0.03$) higher milk yield compared to all the other treatments. No significant difference was observed between the other pasture treatments.

## *Milk fat composition*

The total milk fat (g/kg DM) and milk fatty acid composition (g/100g fatty acids) in milk from cows grazing HS, HSD, RG, RGD, TF and TFD pastures are presented in Table 7. The total fat yield did not differ significantly between simple and diverse pastures. The content of linoleic and linolenic acid was significantly higher in the diverse pastures compared to the simple pastures. Simultaneously there was a significant decrease in the content of *cis*-9, *trans*-11 $C_{18:2}$. The content of *trans*-11 $C_{18:1}$ decreased in all the diverse pasture treatments but the decrease did not reach statistical significance. The content of long chain saturated fatty acids (LCSFA) increased slightly in milk from cows on the diverse pastures but the increase was not significant when all the simple pastures were compared to the diverse ones. The total content of medium chain saturated fatty acids (MCSFA) in milk collected from cows in all treatments did not differ; neither did the total content of monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) or saturated fatty acids (SFA).

*Table 7. Milk yield, milk fat proportion and milk fatty acid composition in milk from cows grazing either a simple or a diverse pasture. The mean values of the all the simple pastures were calculated and compared to the mean values of all the diverse pastures*

| ***Fatty acids (g/100g fatty acids)*** | ***HS*** | ***HSD*** | ***RG*** | ***RGD*** | ***TF*** | ***TFD*** | ***Mean*** | | ***LSD*** | ***P*** |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | ***Simple*** | ***Diverse*** | | |
| Milk yield (kg/d) | 16.85 | 15.40 | 15.79 | 16.78 | 19.18 | 16.73 | 17.27 | 16.30 | 1.69 | n.s. |
| Milk fat (g/kg milk solids) | 332.20 | 341.12 | 341.59 | 347.85 | 341.02 | 321.94 | 338.27 | 336.97 | 39.64 | n.s. |
| Linoleic acid | 0.61 | 0.89 | 0.65 | 0.97 | 0.80 | 1.05 | 0.69 | 0.97 | 0.25 | $<0.05$ |
| Linolenic acid | 0.61 | 0.91 | 0.65 | 0.99 | 0.98 | 1.02 | 0.75 | 0.98 | 0.22 | $<0.05$ |
| $C_{18:1}$ *trans*-11 | 3.24 | 3.12 | 3.20 | 3.00 | 3.15 | 2.37 | 3.20 | 2.80 | 0.65 | n.s. |
| $C_{18:2}$ *cis*-9, *trans*-11 | 1.62 | 1.52 | 1.68 | 1.38 | 1.72 | 1.29 | 1.67 | 1.40 | 0.25 | $<0.05$ |
| LCSFA[1] | 44.79 | 46.03 | 44.95 | 45.56 | 44.12 | 45.93 | 44.59 | 45.84 | 2.62 | n.s. |
| MCSFA[2] | 25.60 | 24.78 | 25.05 | 24.45 | 25.13 | 25.37 | 25.29 | 24.87 | 1.18 | n.s. |
| Fatty acids above 16C | 70.69 | 71.45 | 71.19 | 71.78 | 71.09 | 70.82 | 70.97 | 71.35 | 1.11 | n.s. |
| Fatty acids below 16C | 28.99 | 28.20 | 28.46 | 27.84 | 28.55 | 28.84 | 28.69 | 28.30 | 1.12 | n.s. |
| Total MUFA[3] | 22.95 | 22.00 | 23.05 | 22.50 | 23.02 | 21.32 | 23.02 | 21.94 | 1.90 | n.s. |
| Total PUFA[4] | 4.67 | 5.18 | 4.88 | 5.42 | 5.71 | 5.34 | 5.08 | 5.32 | 0.64 | n.s. |
| Total SFA[5] | 72.06 | 72.48 | 71.71 | 71.70 | 70.91 | 72.96 | 71.55 | 72.38 | 2.37 | n.s. |

n.s.: not significant

[1] Long chain saturated fatty acids ($C_{16:0}$ – $C_{26:0}$); [2] Medium chain saturated fatty acids ($C_{6:0}$ – $C_{15:0}$); [3] Monounsaturated fatty acids; [4] Polyunsaturated fatty acids; [5] Saturated fatty acids.

In order to investigate the effect inclusion of diverse plant species had on milk yield and fatty acid composition each of the diverse pasture mixtures were compared to its simple mixture of grass and white clover. When the fatty acid profile was compared between the HS and the HSD treatments a significant difference was observed in the content of both linoleic and linolenic acid ($P<0.001$) as well as in the content of total PUFA ($P<0.1$) (Table 8). There was also a small decrease in the content of *cis*-9, *trans*-11 $C_{18:2}$ as well as an increase in the content of LCSFA and total content of fatty acids above 16 carbons but none of these values reached statistical significance.

*Table 8. Comparison in milk fatty acid profiles from cows grazing either simple or diverse pastures with the base grass high-sugar perennial ryegrass*

| ***Fatty acids (g/100g fatty acids)*** | ***HS*** | ***HSD*** | ***LSD*** | ***P*** |
|---|---|---|---|---|
| Linoleic acid | 0.61 | 0.89 | 0.10 | <0.001 |
| Linolenic acid | 0.61 | 0.91 | 0.12 | <0.001 |
| $C_{18:1}$ *trans*-11 | 3.24 | 3.12 | 0.83 | n.s. |
| $C_{18:2}$ *cis*-9, *trans*-11 | 1.62 | 1.52 | 0.39 | n.s. |
| LCSFA[1] | 44.79 | 46.03 | 2.68 | n.s. |
| MCSFA[2] | 25.60 | 24.78 | 1.50 | n.s. |
| Fatty acids above 16C | 70.69 | 71.45 | 1.37 | n.s. |
| Fatty acids below 16C | 28.99 | 28.20 | 1.37 | n.s. |
| Total MUFA[3] | 22.95 | 22.00 | 2.09 | n.s. |
| Total PUFA[4] | 4.67 | 5.18 | 0.54 | <0.1 |
| Total SFA[5] | 72.06 | 72.48 | 2.58 | n.s. |

n.s.: not significant

[1] Long chain saturated fatty acids ($C_{16:0}$ – $C_{26:0}$); [2] Medium chain saturated fatty acids ($C_{6:0}$ – $C_{15:0}$); [3] Monounsaturated fatty acids; [4] Polyunsaturated fatty acids; [5] Saturated fatty acids.

There was a significantly higher concentration of PUFA, including linoleic and linolenic acid, in milk from cows grazing the RGD pasture compared to cows on the RG pasture (Table 9). No other values reach statistical significance although a small decrease in the content of *cis*-9, *trans*-11 $C_{18:2}$ can be observed.

*Table 9. Comparison in milk fatty acid profiles from cows grazing either simple or diverse pastures with the base grass standard perennial ryegrass*

| ***Fatty acids (g/100g fatty acids)*** | ***RG*** | ***RGD*** | ***LSD*** | ***P*** |
|---|---|---|---|---|
| Linoleic acid | 0.65 | 0.97 | 0.14 | <0.001 |
| Linolenic acid | 0.65 | 0.99 | 0.18 | <0.001 |
| $C_{18:1}$ *trans*-11 | 3.20 | 3.00 | 0.90 | n.s. |
| $C_{18:2}$ *cis*-9, *trans*-11 | 1.68 | 1.38 | 0.40 | n.s. |
| LCSFA[1] | 44.95 | 45.56 | 2.49 | n.s. |
| MCSFA[2] | 25.05 | 24.45 | 1.79 | n.s. |
| Fatty acids above 16C | 71.19 | 71.78 | 1.77 | n.s. |
| Fatty acids below 16C | 28.46 | 27.84 | 1.76 | n.s. |
| Total MUFA[3] | 23.05 | 22.50 | 1.90 | n.s. |
| Total PUFA[4] | 4.88 | 5.42 | 0.55 | <0.1 |
| Total SFA[5] | 71.71 | 71.70 | 2.24 | n.s. |

n.s.: not significant
[1] Long chain saturated fatty acids ($C_{16:0}$ – $C_{26:0}$), [2] Medium chain saturated fatty acids ($C_{6:0}$ – $C_{15:0}$); [3] Monounsaturated fatty acids; [4] Polyunsaturated fatty acids; [5] Saturated fatty acids.

Table 10 shows the comparison in milk fatty acid profiles from cows on either the TF or the TFD treatment. There was a significant difference in the concentration of linoleic acid and in the total content of MUFA with a higher and a lower content in the milk from cows grazing the diverse pasture, respectively. There was a tendency of significance difference in the content of *trans*-11 $C_{18:1}$, as well as in the content of LCSFA and total SFA. Unlike the other treatments the concentration of linolenic acid did not increase but was at a relatively high level in the milk from cows on both treatments.

*Table 10. Comparison in milk fatty acid profiles from cows grazing either simple or diverse pastures with the base grass tall fescue*

| ***Fatty acids (g/100g fatty acids)*** | ***TF*** | ***TFD*** | ***LSD*** | ***P*** |
|---|---|---|---|---|
| Linoleic acid | 0.80 | 1.05 | 0.15 | <0.01 |
| Linolenic acid | 0.98 | 1.02 | 0.17 | n.s. |
| $C_{18:1}$ *trans*-11 | 3.15 | 2.37 | 0.84 | <0.1 |
| $C_{18:2}$ *cis*-9, *trans*-11 | 1.72 | 1.29 | 0.54 | n.s. |
| LCSFA[1] | 44.12 | 45.93 | 2.00 | <0.1 |
| MCSFA[2] | 25.13 | 25.37 | 1.14 | n.s. |
| Fatty acids above 16C | 71.09 | 70.82 | 1.15 | n.s. |
| Fatty acids below 16C | 28.55 | 28.84 | 1.15 | n.s. |
| Total MUFA[3] | 23.02 | 21.32 | 1.58 | <0.05 |
| Total PUFA[4] | 5.71 | 5.34 | 0.70 | n.s. |
| Total SFA[5] | 70.91 | 72.96 | 2.17 | <0.1 |

n.s.: not significant
[1] Long chain saturated fatty acids ($C_{16:0}$ – $C_{26:0}$); [2] Medium chain saturated fatty acids ($C_{6:0}$ – $C_{15:0}$); [3] Monounsaturated fatty acids; [4] Polyunsaturated fatty acids; [5] Saturated fatty acids.

# Discussion

## *Milk yield*

The daily milk yield did not differ significantly between cows on the simple compared to the diverse pastures, and there does not seem to be any beneficial effect of inclusion of diverse plant species on the milk production. However, the milk production from cows on the TF pasture was significantly higher than the production from cows on the TFD pasture (P = 0.03). This might be due to the fact that tall fescue is a good summer crop in dry environments, such as in Canterbury, as it has higher water use efficiency than ryegrasses and may produce more kg dry matter per hectare, especially if the weather is hot and dry (Millée *et al.*, 2010). The TF pasture also had a much higher content of crude protein (19.44%). This might have some contributing effect although many studies have shown no positive effect of a crude protein content higher than 16% of DM on milk production (Kalscheur *et al.*, 1999; Colmenero & Broderick, 2006). Other explanations to the higher milk yield from cows on the TF pasture is a possibly higher intake of dry matter or the fact that the proportion of dead plant material was much lower in that pasture compared to both HS, HSD and RG. The TF pasture was also the only pasture that contained a relatively high proportion of white clover (23.79%). It is well known that the clover content of the pasture has an impact on the milk production as a higher content of clover increase the utilisation efficiency of amino acids in the animal (Johnson & Thomson, 1996). The combination of the drought resistant tall fescue with white clover in the pasture mixture would therefore result in a highly nutritious pasture for the dairy cows, especially if the weather was dry during the experimental period which might have been the fact considering the large proportions of dead plant materials in some of the pastures.

Cows on the high sugar ryegrass pasture (HS) had a numerically higher daily milk production compared to cows on the standard ryegrass pasture (RG). These results correspond to other studies where diets consisting of high sugar ryegrasses resulted in an increased milk production (Miller *et al.*, 2001b). Although different studies have come to varying conclusions, ryegrasses with higher sugar content have been shown to increase milk production by improving ruminal amino acid utilisation for microbial protein synthesis as a result of a better supply of easily available carbohydrates, e.g. WSC (Moorby *et al.*, 2006). Although the WSC content did not differ between the pastures the HS pasture had a higher digestibility of organic matter (OMD) than the RG and higher protein content (16.21 vs. 13.41%). However, when including more plant species in the pasture the RGD treatment resulted in higher milk yield compared to the HSD treatment. This might be a consequence of the lower proportion of high sugar ryegrass in the pasture at the expense of more plant species or the fact that the RGD pasture had higher crude protein content (16.24 vs. 13.67%) as well as a higher proportion of red clover (24.20 vs. 0.85% of DM). The lower production in cows grazing HSD and RG pastures may also be a consequence of the low clover content in those pastures as Cosgrove *et al.* (2006) stated that a common problem with diverse pastures is that they often contain insufficient amounts of clover to capture their nutritional value as extra milk production. Since several of the pastures had a low inclusion level of white clover this might be a likely explanation to the lower milk production in cows grazing these pastures.

## *Milk fatty acid composition*

Before discussing the changes in milk fatty acid composition it should be mentioned that there is a chance that the experimental period in this study was too short for the fatty acid composition to change and establish properly according to the composition of the different pasture treatments. The adaptation period in this study was only four days and the milk samples were collected on day six in the experiment which leaves little time for changes in the milk composition to take place. Khanal *et al.* (2008) investigated the changes in fatty acid composition after cows were turned out to pasture and measured how many days it took for the different fatty acids to stabilise their concentration in the milk. According to their results it took around 23 days for most of the fatty acids in the milk to get stabilised after the cows were turned out to pasture but only seven days for the values to be back at their original level when the cows were put back on a TMR diet inside the barn. Since the milk samples in this study were obtained on day six the fatty acid profile might not yet have been stabilised to the final level. The same quick changes in the milk fat composition in cows after transition from a fresh grass diet to a silage diet have been observed by Elgersma *et al.* (2004) as well. They observed that most changes in milk fatty acid composition took place within four days after transition to the silage diet It should, however, be mentioned that these experiments compared the fatty acid composition in milk from cows on either a pasture or a silage diet. No data have been found on how long it takes for the fatty acid composition to stabilise after cows have been subjected to changes in their pasture diet. Nonetheless, in the present study, in spite of the short length of the experimental period, some differences in milk fatty acid composition among pastures types were evident.

All the diverse pasture treatments resulted in a significantly higher content of the PUFA linoleic and linolenic acid in the milk compared to the simple mixtures (Table 7). Meanwhile, the concentration of *cis*-9, *trans*-11 $C_{18:2}$ was significantly lower in the milk from cows on these treatments, which differs from the hypothesis stated in the introduction. The concentration of linolenic acid in milk fat increased in each diverse pasture mixture compared to the simple mixtures, except for cows grazing the TFD pasture where the increase was not significantly higher than in milk from cows grazing the TF pasture. A possible explanation to the smaller increase in linolenic acid between these treatments is that the concentration of this fatty acid was high in milk from cows on both treatments (~1 g/100g fatty acids). The TF pasture appears to result in a high content of linolenic acid in milk, similar to the increases observed in milk from cows grazing diverse pastures. Therefore the difference in linolenic acid content between TF and TFD is not as large as it is between the other treatments.

There was a small increase in the concentration of LCSFA in the milk from cows on the diverse pastures but the increase did not reach statistical significance, except for between the TF and TFD pastures. Simultaneously there was a small decrease in the proportion of MCSFA in most of the diverse pasture treatments which indicates lower *de novo* synthesis. According to previous studies feeding ruminants diverse forage diets result in higher concentrations of both linoleic and linolenic acid in the milk as well as increased concentrations of *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 $C_{18:2}$ (Kraft *et al.,* 2003; Lourenço *et al.,* 2005b). However, Lourenço *et al.* (2008) compiled statistical data from several studies on effects of diverse forages on the milk fatty acid profiles and stated that the concentration of *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 $C_{18:2}$ varied between the studies. They suggested that the concentration of these fatty acids in milk depends of several different factors and stated that no certain correlation can be determined between feeding diverse plant species and the concentration of *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 $C_{18:1}$ in milk fat.

The significant increase in linoleic and linolenic acid in milk from cows grazing the diverse pastures found in this study is most likely a result of changes in the rumen environment. Different microbial populations have been observed in the rumen of cows receiving different diets which might explain the changes in the biohydrogenation intermediates as well as in the amount and proportion of fatty acids escaping the rumen and ending up in the milk (Lourenço *et al.,* 2007a). Lourenço *et al.* (2007b) also found that the abomasum content of both linoleic and linolenic acid was significantly higher in lambs grazing a botanically diverse pasture, although this difference could not be observed in the rumen contents which indicate a higher rumen outflow of these fatty acids. Lourenço *et al.* (2005b) suggested that the higher concentrations of PUFA in milk from cows fed diverse diets were due to higher transfer efficiency from the duodenum to the mammary gland of these fatty acids**.** This statement has later been supported by Peterson *et al.* (2011). Furthermore, several studies have found correlations between the microbial activity in the rumen and feeding diverse plant species to ruminants (Lourenço *et al.,* 2007a; Lourenço *et al.,* 2007b). The most likely explanation for the changes that occur in the milk fatty acid profiles from cows receiving diverse diets is that the microbial population in the rumen is affected by different secondary metabolites in the diverse plant species which result in a reduced lipolysis and hydrogenation of linoleic and linolenic acid (Lourenço *et al.,* 2007b). These suggestions are based on the demonstrated effects some secondary plant metabolites have on rumen methanogenesis (Waghorn, 2008) but the mechanism behind it is not yet fully understood. Another explanation to the higher concentrations of linoleic and linolenic acid might be that the different pasture mixtures differed in concentrations of these fatty acids as correlations have been found between biohydrogenation patterns and the content of PUFA in plant species (Collomb *et al.,* 2002b).

The higher concentration of linoleic and linolenic acid in milk from cows subjected to the RGD treatment compared to the RG treatment might in part be explained by the inclusion of red clover in the diverse pasture mixture. Inclusion of red clover in the diet has been shown to result in higher concentrations of linoleic and linolenic acid in the milk (Dewhurst *et al.,* 2003; Lourenço *et al.,* 2008; Van Ranst *et al.,* 2011; Arvidsson *et al.,* 2012) as well as a lower biohydrogenation of these fatty acids in the rumen (Van Ranst *et al.,* 2011). Lourenço *et al.* (2008) compiled results from different studies where cows were fed red clover forages compared to ryegrass forages. The milk from cows fed red clover had higher proportions of linolenic acid, despite the similar dietary supply from both diets, and the rumen proportion and outflow of this fatty acid was also higher. This is a consequence of the fact that red clover contains the plant metabolite polyphenol oxidase (PPO) which is known to reduce the activity of plant lipases (Lourenço *et al.,* 2005a; Lee *et al.,* 2007). It acts by forming diphenols from phenols, which are both present in plants, and then oxidising the diphenols to quinones. The quinones react with each other and form a polymer network which can also bind to certain amino acids (e.g. lysine, cysteine, and methionine), thereby including proteins in their network. As a consequence, the proteins are denatured and enzyme activity is inhibited which might be an explanation for the lower biohydogenation (Lourenço *et al.,* 2008; Van Ranst *et al.,* 2011). The quinones may also react with microbial enzymes in the rumen, thereby inhibiting the lipolysis. This is, however, not as likely as the quinones are very reactive and would have bond to proteins or other quinones before reaching the rumen. Nevertheless, Lee *et al.* (2007) concluded that the presence of PPO in forages have a direct impact on biohydrogenation. In their study two lines of red clover were compared; containing high and low concentrations of PPO, respectively. They found that the biohydrogenation of linoleic and linolenic acid was significantly lower in the treatment with high concentrations which suggests that forages high in PPO activity may be used to reduce the losses of PUFA in the rumen. Another explanation to the lower biohydrogenation is that the quinones can bind to the lipid membranes, protecting them from lipolysis (Lee *et al.,* 2007; Lourenço *et al.,* 2008). Since the accumulation of unesterified linoleic and linolenic acid is limited in the rumen lower lipolysis will result in a higher outflow of these fatty acids which will end up in the milk to a higher extent (Harfoot & Hazlewood, 1997; Lourenço *et al.,* 2008).

The increase of linoleic and linolenic acid in milk from cows grazing the diverse pastures might also be a consequence of the higher concentration of CT in those diets. As is shown in Table 1 chicory and plantain contain higher levels of CT than grasses. Chicory and plantain were present in all the diverse mixtures, along with big trefoil in the TFD treatment. CT act by binding to proteins in the rumen, forming non-degradable complexes, resulting in a higher flow of non-ammonia nitrogen to the duodenum (Waghorn *et al.,* 1994). The bindings within the complexes are pH dependent and since the pH in the duodenum is higher the tannin-protein complexes are broken and the proteins can be absorbed through the intestinal wall. Thus, less PUFA are subjected to biohydrogenation by the rumen bacteria and may instead end up in the milk in a higher amount. CT may also affect the microbial population in the rumen either indirectly, by affecting the microbial enzymes and inhibiting their activity (McSweeney *et al.,* 2001), or directly by altering the permeability of their membranes and inhibiting their oxidative phosphorylation (Scalbert, 1991). Inclusion of moderate amounts of CT in the ruminant diet (<50 g/kg DM) has been shown to have beneficial effects by improving the digestibility and utilisation of the feed by the animal (Barry & McNabb, 1999; Min *et al.*, 2003; Frutos *et al.*, 2004). Very high levels of CT, as in big trefoil (77 g/kg DM; Ramírez-Restrepo & Barry, 2005) might have a negative effect on the cow's performance with consequences as reduced voluntary feed intake and reduced nutrient availability to the animal (Barry & Duncan, 1984; Barry, 1985). However, in this experiment the proportion of diverse plant species containing CT were relatively small in each of the diverse pasture mixtures, thus no such negative effect would be expected. In fact the proportion of CT might even be too small (<10 g/kg DM) for a beneficial effect to be noticeable. Both chicory and plantain contain relatively low concentrations of CT (see Table 1) and according to the values presented in this study the proportion of CT did not exceed 3.5 g/kg DM for any of the pasture mixture at the time of sampling. The concentrations of CT in the pastures included in this study might therefore be too low to get a positive effect on the milk composition. There is also a difference in the reactivity of CT between plant species and the effect depends both upon its concentration and chemical structure (Min *et al.,* 2003). It is therefore possible that the CT present in chicory and plantain do not have any substantial impact on the milk production and milk composition when fed to dairy cows. Furthermore, Beever and Siddons (1986; cited by Barry & McNabb, 1999) suggested that mixing CT containing plant species with non-CT containing species seldom results in any beneficial effects since the CT will preferably react with the proteins from the CT-containing plant. There is therefore a chance that the CT in the diverse pastures in this study did not contribute to the positive effects seen in the milk fatty acid profile from cows on these pastures.

Milk from cows grazing diverse pastures had significantly lower concentrations of *cis*-9, *trans*-11 $C_{18:2}$ compared to cows grazing the simple pastures. However, the milk from all cows in the study had a rather high concentration of this fatty acid compared to other studies (0.87g/100g; Collomb *et al.*, 2002a). In other studies where the *cis*-9, *trans*-11 $C_{18:2}$ content decreased in milk from cows fed botanically diverse diets the concentration in the milk was already high which might explain the lack of effect on the composition of this fatty acid (1.7 g/100g; Leiber *et al.,* 2005). The lower concentration of *cis*-9, *trans*-11 $C_{18:2}$ might also be a consequence of the higher concentrations of linoleic and linolenic acid in the milk. As already been discussed the PPO in red clover may protect the lipids from lipolysis in the rumen resulting in a higher outflow of these fatty acids and thus a lower biohydrogenation of them. Since *cis*-9, *trans*-11 $C_{18:2}$ is an intermediate in the biohydrogenation of linoleic and linolenic acid (as is *trans*-11 $C_{18:1}$) the outflow might be lower if less linoleic and linolenic acid is hydrogenated.

Besides the possible impact different plant secondary metabolites may have on the milk fatty acid composition the composition of the pastures may have further impact on the milk fatty acid profile, especially the concentrations of WSC and protein. A high WSC content leads to a decreased pH in the rumen which will affect the microbial population and hence the biohydrogenation of the unsaturated fatty acids, whilst an increased protein content will result in higher pH (Eeston *et al.*, 2009). The relationship between WSC and CP was negative for the RGD, TF and TFD pastures but positive for the HS, HSD and RG pastures. However, the concentration of linoleic and linolenic acid was somewhat higher for the RGD, TF and TFD pastures compared to the others which would not be expected since the rumen pH would have been higher in the rumen of the cows grazing these pastures. Nevertheless, the proportion of dead plant material in these pastures were much lower compared to the other pastures which possibly had a larger effect on the milk fatty acid composition than the content of WSC.

Finally, one important detail to bear in mind when studying the effects of botanically diverse diets on milk composition is that the term “botanically diverse” is not a fixed concept. Thus the diversity and number of plant species in the diet may vary between studies which will have an impact on the results. In this study the diverse pasture mixtures only contained three or four extra species and may not be considered to be “diverse” in comparison to other studies or in other parts of the world, although the inclusion levels of diverse plant species in this study were rather high. In a study by Leiber *et al.* (2005) 71 plant species were used in the pasture mixture, of which 46 were herbs. Other studies, by Collomb *et al.* (2002a,b) and Lourenço *et al.* (2005b), used 55 and 42 plant species in their pastures of which 13 and 22 were herbs, respectively. However, the proportion of diverse plant species in the pasture does not seem to have a direct impact on the milk fatty acid profile as Leiber *et al.* (2005) reported lower content of *cis*-9, *trans*-11 $C_{18:2}$ in milk although their pasture contained more plant species than the other studies listed above in which the concentration of *cis*-9, *trans*-11 $C_{18:2}$ increased in the milk. There seems to be an interaction of several different factors that affect and determine the fatty acid profile in milk which implies that further research within the area is essential.

# Conclusions

Feeding botanically diverse pastures to dairy cows affect the milk fat fatty acid proportions, increasing the proportions of PUFA, especially of linoleic and linolenic acid. The higher transfer efficiency of these fatty acids from feed to milk is probably caused by changes in the rumen environment due to various plant metabolites present in herbs and legumes that have antimicrobial activity and may interfere with the biohydrogenation in the rumen and the microbial population.

Including more diverse plant species in the ruminant diet provides an opportunity to produce healthier products from a human perspective with higher concentrations of unsaturated fatty acids. This, along with the reported decreases in rumen methanogenesis from cows fed diverse forages, indicates that the effect of secondary plant metabolites on ruminant production merit further investigation.

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|---|---|---|
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