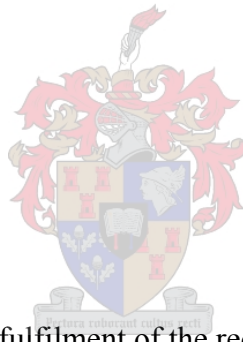# Effects of nutrition on the Conjugated Linoleic Acid content of milk

Lindie Liebenberg

Thesis presented in partial fulfilment of the requirements for the degree of

Master of Science in Agriculture
(Animal Sciences)

at Stellenbosch University

Supervisor: Prof . C.W. Cruywagen

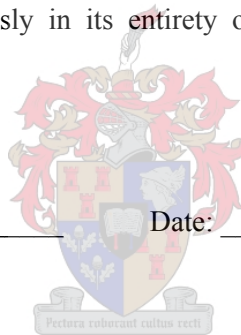Stellenbosch   March 2007

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any other university for a degree.

Signiture: _____ Date: _____

Summary

The objective of this investigation was to determine the effect of supplemented conjugated linoleic acid (CLA) on milk production and milk composition of lactating dairy cows in production systems commonly used in South Africa.

In the first of three trials, samples of 24 bulk tanks were collected to get an impression of the CLA status of milk in the Western Cape Province, South Africa.   Six samples were each collected from Jersey and Holstein herds in total mixed ration (TMR)-based systems. Another six samples were each collected from Jersey and Holstein herds on pasture-based systems. An additional three samples were also collected from co-op silos. One of these came from a mixed herd on TMR, one from a Jersey herd on TMR and the third sample from a mixed herd on pasture. The CLA levels of the milk were within the range reported in literature, albeit on the low side. The mean CLA level in milk fat of cows from pasture and TMR-based systems were 10.5 and 5.45 mg/g of fatty acids, respectively.   This is in agreement with trends reported in the literature with cows on pasture having higher levels of CLA in their milk than cows on TMR's. In this study, breed had no effect on the CLA level in milk fat.

In the second trial, forty multiparous lactating Jersey cows were used in a pasture based experiment to determine effects of a commercial CLA supplement on the CLA content of milk fat. The cows in the two groups (n=20) received 6 kg concentrate per day, with the cows in the CLA treatment group receiving an additional 70 g of the CLA supplement, which was hand mixed into the concentrate just before feeding. The CLA treatment had no effect on milk yield, protein content, lactose content or milk urea nitrogen (MUN). However, supplemental CLA resulted in milk fat depression which can be ascribed to the presence of *trans*-10*, cis*-12 $C_{18:2}$ CLA in the supplement. There was a 1.4 fold increase in *cis*-9*, trans*-11 $C_{18:2}$ CLA (rumenic acid) concentrations in the CLA treatment group.

The third trial involved 12 multiparous lactating Holstein cows which were used in a 4 x 4 Latin square (n=3) design to determine the response of an enhanced supply of polyunsaturated fatty acids and the CLA supplement on milk production and fatty acid composition. The cows received a restricted amount of TMR, calculated to supply the required energy and protein requirements and to maintain milk production. The four diets consisted either of a basal control diet, basal diet + 1% tuna oil, basal diet + 1% CLA supplement, or basal diet + 0.5% tuna oil and 0.5% CLA supplement. Milk production, protein, lactose and MUN contents were unaffected by diet. However, milk fat percentage and milk fat yield were

decreased by the treatments. The CLA treatment alone resulted in the highest rumenic acid (RA) concentration in the milk fat. However, the combined treatment of tuna oil and CLA, as well as the tuna oil alone, also resulted in higher RA values compared to the control treatment.

Results indicated that the CLA content of milk fat can be manipulated by use of CLA supplements and tuna oil to improve the wholesomeness of milk to human consumers.
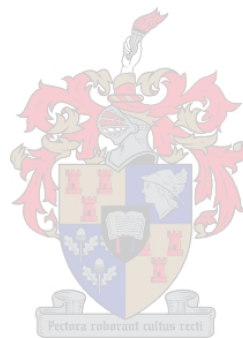
Opsomming

Die doel van hierdie studie was om die effekte van 'n CLA-supplement op die melkproduksie en melksamestelling van lakterende melkkoeie in algemene produksiesisteme in Suid-Afrika te ondersoek.

In die eerste van drie proewe is melkmonsters van 24 massatenks versamel om 'n indruk te kry van die CLA-status van melk in Suid-Afrika, meer spesifiek in die Wes-Kaapprovinsie. Ses monsters elk is van Jersey- en Holsteinkuddes in volvoerstelsels versamel. 'n Verdere ses monsters elk is versamel van Jersey- en Holsteinkuddes op weiding. Daarbenewens is drie monsters van ko-operatiewe silos in die Wes-Kaap verkry. Een van hierdie drie monsters is van 'n gemengde kudde op volvoer geneem, een van 'n Jerseykudde op volvoer en een van 'n gemengde kudde op weiding. Die CLA-inhoud van die melk was binne die grense van dit wat in die literatuur gerapporteer is, hoewel aan die lae kant. Gemiddelde CLA-inhoud van melkvet afkomstig van koeie op weiding en koeie op volvoer was onderskeidelik 10.5 en 5.45 mg/g vetsure. Die CLA-inhoud was hoër in melk afkomstig van koeie op weiding as dié van koeie op volvoer. Dit is in ooreenstemming met die neiging wat in die literatuur gerapporteer is, naamlik dat koeie op weiding hoër CLA-vlakke in hul melk toon as koeie op volvoerdiëte. In hierdie studie het ras geen effek op die CLA-vlakke in die melkvet gehad nie.

In die tweede proef is 40 lakterende Jerseykoeie op weiding gebruik om die effekte van 'n kommersiële CLA-supplement op die CLA-inhoud van melkvet te ondersoek. Koeie in die twee groepe (n=20) het elk 6 kg konsentraat per dag ontvang en koeie in die CLA-behandeling het elk 70 g van die CLA-produk ontvang wat net voor voeding per hand ingemeng is. Die CLA-behandeling het geen effek op melkopbrengs, proteïeninhoud, laktose-inhoud of melk-ureumstikstof gehad nie. Die CLA-behandeling het egter bottervetdepressie tot gevolg gehad wat toegeskryf kan word aan die teenwoordigheid van *trans*-10, *cis*-12 $C_{18:2}$ CLA in die CLA-supplement. Daar was 'n 1.4 x toename in *cis*-9, *trans*-11 $C_{18:2}$ CLA (rumensuur) in die CLA-behandelingsgroep.
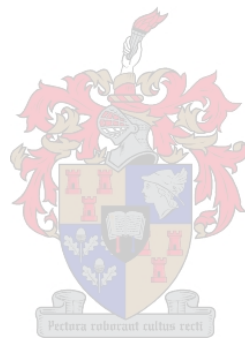
Die derde proef het 12 lakterende Holsteinkoeie ingesluit wat in 'n 4 x 4 Latynse vierkantontwerp (n=3) gebruik is om die effekte van 'n verhoogde voorsiening van poli-onversaadigde vetsure en CLA op die melkproduksie en vetsuursamestelling te ondersoek. Die koeie het 'n beperkte hoeveelheid volvoer ontvang om aan die energie- en proteinbehoeftes te voldoen en melkproduksie te onderhou. Die vier diëte het bestaan uit 'n basale kontroledieet, basale dieet + 1% tuna-olie, basale dieet + 1% CLA-supplement en 'n

basale dieet + 0.5% tuna-olie en 0.5% CLA-supplement. Daar was geen verskille tussen behandelings ten opsigte van melkproduksie nie. Die proteïeninhoud, laktose-inhoud en melk-ureumstikstof is ook nie beïnvloed nie. Behandelings het 'n daling in melkvetpersentasie en melkvetopbrengs tot gevolg gehad. Die CLA-behandeling alleen het die hoogste rumensuur (RS) konsentrasie in die melkvet tot gevolg gehad. Die kombinasiebehandeling van van tuna-olie en CLA-supplement, asook die tuna-olie alleen, het egter ook die RS-inhoud verhoog in vergelyking met die kontrolebehandeling.

Resultate van hierdie reeks proewe het getoon dat die CLA-inhoud van melk deur die gebruik van CLA-supplemente en tuna-olie gemanipuleer kan word om die heilsaamheid van melk te verhoog.
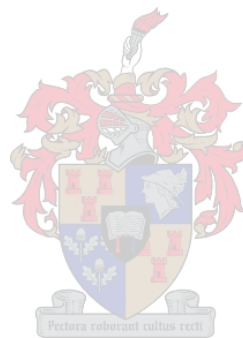
I dedicate this thesis to my parents, Johan and Marinda Pienaar who through the years taught me the meaning of searching for wisdom, truth and understanding in the word of God.

Acknowledgements

My husband, Izak, for continuously supporting me, for being my friend, and for encouraging me when my spirits were low;

My family and friends for their prayers and support;

My Heavenly Father, for giving me life, faith and hope, and for giving me strength to live life.
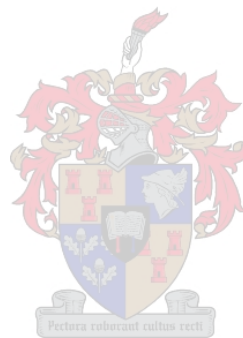
Notes

The language and style used in this thesis are in accordance with the requirements of the *South African Journal of Animal Science.* This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

Abbreviations

ADF   Acid detergent fibre

Ca   Calsium

CF   Crude Fat

CLA   Conjugated linoleic acid

CP   Crude protein

Cu   Copper

DIM   Days in milk

DM   Dry matter

DMI   Dry matter intake

EE   Ether extract

FA   Fatty acid

LA   Linoleic acid

LNA   Linolenic acid

MFD   Milk fat depression

MUN   Milk urea nitrogen

MP   Milk production

NDF   Neutral detergent fibre

NSC   Non- structural carbohydrates

PUFA   Polyunsaturated fatty acids

RA   Rumenic acid

SEm   Standard error of mean

TMR   Total mixed ration

VA   Vaccenic acid

Contents

CHAPTER 1

General introduction

Conjugated linoleic acid (CLA) is a fatty acid found predominantly in products, such as milk and meat, of ruminant origin. Milk and milk products are the richest source of CLA that are both accessible and acceptable to most consumers. With the large variety of milk products consumed on a daily basis CLA is provided naturally to the consumer.

In the past decade CLA have received a considerable amount of attention due to the identification of certain important positive properties that CLA might exert on human and animal health. A number of studies conducted on cell cultures, animal models and some human studies found CLA to be a potent anticarcinogen, as well as having antiatherogenic, immune-modulating, anti-diabetic and cholesterol lowering properties and other health benefits. It was confirmed that the daily intake of CLA that would provide cancer protection, is higher than the CLA that is currently being consumed by the average person.

Conjugated linoleic acid is produced naturally in the ruminant from dietary linoleic, linolenic, and *trans*-11 $C_{18:1}$ fatty acids (FA). Synthesis of CLA occurs either in the rumen during ruminal biohydrogenation of FA or in the tissues by Δ^9 desaturase enzyme activity. By increasing intake and the flow of these FA, it is possible to increase the production of CLA in the animal. With increased production of CLA there would be an increase of CLA in milk and therefore, human CLA consumption will increase without the need to consume more or larger portions of milk products.

This project consists of three studies. The first of one was undertaken as a survey to determine the CLA levels of milk produced from two production systems used in South Africa and two different breeds. The two systems studied were a total mixed ration (TMR) system and a pasture system. On pasture systems the diets are generally supplemented with concentrates to ensure sufficient intake of energy and protein. Milk samples were collected from Jersey and Holstein herds within these systems, as well as a mixture of both breeds within each production system.

Secondly, the effect of a commercial CLA supplement, mixed into a commercial concentrate and fed as supplement on summer pasture, was investigated. It has been well documented that levels of CLA can be increased up to seven fold on lush pasture. Within this study, the

effect of a commercial CLA supplement over and above the pasture effect on the CLA content of milk was quantified.

The last experiment was undertaken to investigate the effects of a commercial CLA supplement, tuna fish oil and a combination of the two on CLA in milk fat. For this experiment a TMR production system was used. It has been shown in the literature that a combination of products may have an additive effect on the CLA content of milk.
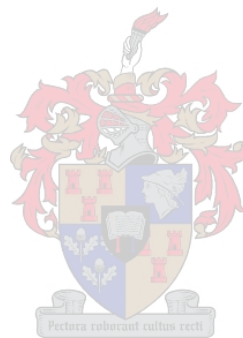
CHAPTER 2

Literature review

2.1   Introduction

Conjugated Linoleic Acid (CLA) is the collective term used to describe the geometric (*cis/cis, cis/trans, trans/cis, trans/trans*) and positional (double bond position 7 & 9; 8 & 10; 9 & 11; 10 & 12; 11 & 13; 12 & 14) isomers of octadecadienoic acid ($C_{18:2}$) (Aydin *et al.,* 2005; Rickert *et al.,* 1999; Stanton *et al.,* 1997) containing a conjugated unsaturated double bond system, which consists of two double bonds, separated by single carbon – carbon bonds instead of a methylene group (Chin *et al.,* 1992; Dhiman *et al.,* 1999a,b; Parodi., 1999; Dhiman *et al.,* 2000; Donovan *et al.,* 2000; Griinari *et al.,* 2000; Dugan *et al.,* 2001; Abu-Ghazaleh *et al.,* 2002; Parodi., 2003). CLA is a group of unsaturated fatty acid isomers that occur naturally in foods derived from ruminants, and is found in its highest concentration in bovine milk (Chin *et al.,* 1992; Dhiman *et al.,* 1999; Kelsey *et al.,* 2003; Aydin *et al.,* 2005;). Unless otherwise specified, CLA indicates a mixture of isomers. CLA is therefore a category and *cis*-9, *trans*-11 $C_{18:2}$ CLA is a unique molecule in that category. This is important as different isomers have different attributes and some, unlike others, may be beneficial for animal and human health (Kelly. 2001). The average CLA content of milk in the USA varies between 3 and 6 mg/g of the total FA (Dhiman *et al.,* 2000). The *cis*-9, *trans*-11 $C_{18:2}$ fatty acid, also termed rumenic acid (Kramer *et al.,* 1998; Ellen & Elgersma, 2004; Destaillats *et al.,* 2005) is the most biologically active and also the most abundant natural isomer of $C_{18:2}$ and accounts for more than 94 % of the CLA in dairy products (Dugan *et al.,* 2001; Collomb *et al.,* 2004; Dhiman *et al.,* 2005). According to Pariza (1999) there is emerging evidence that rumenic acid (RA) and *trans*-10, *cis*-12 $C_{18:2}$ CLA isomers may be responsible for different biological effects, and in some cases they may have a cumulative effect.

2.2   The importance of CLA for Human consumption.

The interest in CLA research has increased in the past few years due to reports of several animal and *in vitro* studies, indicating that consumption of CLA may have health benefits in humans, ranging from cancer prevention to control of type II diabetes (Bauman *et al.,* 2001; Parodi, 2003).

Despite the immense expenditure performed on cancer research during the past three decades, and outstanding progress made in this field, the death rate for cancer patients with invasive and metastatic carcinoma of the colon, breast, lung, pancreas, prostate and bladder, have not decreased very much. Thus prevention rather than therapy should be an important strategy for conquering cancer, and the use of therapeutic foods such as CLA enriched milk can be part of a cancer prevention approach.

Conjugated linoleic acid has been found to be a potent anticarcinogen (Stanton *et al.*, 1997; Aydin *et al.,* 2005). The National Academy of Sciences has pointed out that CLA is the only fatty acid that has been shown unequivocally, to suppress carcinogenesis in experimental animals (Chin *et al.,* 1992; Kalscheur *et al.,* 1997; Ip *et al.*, 1999; Weiss *et al.*, 2004a,b) and inhibit the growth of a large selection of human cancer cell lines *in vitro* (Parodi, 1999). The anticancer effect found with consumption of CLA is the most extensively investigated of all the health benefits that have been identified. Some studies tried to establish diet as an effective route to provide cancer protection (Bauman *et al.,* 2001). In these trials, it was found that there was a direct proportional relationship in the magnitude of the reduction in mammary tumors and the amount of CLA consumed.

Apart from being an effective anticarcinogen, CLA also has numerous properties that could be beneficial to humans and include: antiatherogenic, immunomodulating, growth promoting, and lean body mass-enhancing properties, normalization of impaired glucose tolerance in noninsulin-dependent diabetes, modulating food allergic reactions, the reduction in growth of melanoma, leukaemia, mesothelioma, and glioblastoma together with breast, prostate, colon, and ovarian cancer as well as two human hepatoma cell lines (Parodi, 1999; Cook *et al.*, 2003; Selberg *et al.,* 2004; Weiss *et al.*, 2004a, b; Aydin *et al.,* 2005).

It is indicated by Pariza (1997; 1999), that CLA helps prevent Arteriosclerosis, helps to lower high density lipoprotein while raising plasma low density lipoprotein cholesterol levels in rabbits and it has been shown to affect body composition by reducing the body fat and increasing the muscle and water weight (Dugan *et al,* 2001). In trials by Cook *et al*. (1995), animals fed on diets high in CLA had protection against the catabolic affects of exposure to endotoxins. Animals also had an enhanced immune response and the CLA also served as a growth factor for the young animals.

According to Kelly (2001), Gregory (2001) and Larsen *et al.* (2003), conflicting results have been found on the effects that CLA might have on the human body in relation to lipodystrophy and insulin resistance. It is still noteworthy to conduct more research on the

possibility of, for example, the anticancer and the cholesterol lowering effects on humans that CLA has proven to exhibit.

To experience the positive response of CLA on human health, it has to be consumed in sufficient quantities (Parodi., 2003; Weiss *et al*., 2004b). According to Kelly *et al.* (1998a) the anticarcinogenic effects of CLA occur at low dietary concentrations, the current average intake of humans is close to the dietary level (Ma *et al*., 1999; Parodi., 1994) that demonstrates anticarcinogenic effects in experimental animal models. It is possible to increase the intake of CLA by consumption of foods of ruminant origin, or by increasing the CLA content of milk and meat. The latter approach is more practical since it can be manipulated through the diet of the ruminant. Therefore increasing the CLA content of milk has the potential to increase the nutritive and therapeutic value of milk.

Milk fat CLA is almost entirely RA, which makes milk the most natural source of CLA, with reported values ranging from 2.4 – 28.1 mg/g FA (Parodi, 1997). Ip *et al*. (1994) suggested that an intake of 3.5 g CLA /day for a 70 kg person should be sufficient for cancer prevention. According to Dhiman *et al.* (2005) whole milk contains on average 3.5 % milk fat of which 0.5 % is CLA. Therefore, one serving (227 ml) of whole milk and one serving of cheese (30 g) can provide 90 mg of CLA. This is only 25 % of the amount suggested by Ip *et al*. (1994). However, Knecht *et al.* (1999) found that the risk of breast cancer was halved in women who consumed more than 620 ml of milk per day, compared to those consuming less than 370 ml a day. It is evident from the table below that products from ruminant animals contain the highest concentrations of CLA, of which milk products rank the highest.

Table 2.1 Food sources of CLA. Adapted from Chin *et al*. (1992)

Food source	Content mg/g fat	% Rumenic acid
Condensed milk	7	82
Milk fat	5.5	92
Buttermilk	5.4	89
Lamb	5.4	92
Processed cheeses	5	83
Butter	4.7	88
Ice cream	3.6	86
Other dairy products	3.6-7	82
Natural cheeses	2.9-7.1	83
Yoghurt	1.7-4.8	82
Veal	2.7	84
Turkey	2.5	76
Chicken	0.9	84
Pork	0.6	82
Egg yolk	0.6	82
Shrimp	0.6	n.d.[1]
Non dairy dessert	0.6	n.d.[1]
Trout	0.5	n.d.[1]
Mussels	0.4	n.d.[1]
Formula milk	0.3-0.7	n.d.[1]
Salmon	0.3	n.d.[1]
Sea scallops	0.3	n.d.[1]
Plant oils	0.1-0.7	43

[1]not detectable

2.3   Synthesis of CLA in ruminants.

CLA is formed by the rumen gut micro organisms by microbial isomerization of dietary linoleic acid and desaturation of oleic acid derivatives. The two known pathways for synthesis of CLA are in the rumen or in the tissues. In the rumen, CLA is an intermediate in the biohydrogenation of linoleic acid from dietary fat by the rumen bacteria *Butyrivibrio fibrisolvens* and, in the tissues, CLA is synthesized by Δ^9 desaturase from vaccenic acid (*trans*-11 $C_{18:1}$) (Ellen & Elgersma, 2004; Destaillats *et al.,* 2005). Vaccenic acid (VA) is an intermediate in ruminal biohydrogenation of linoleic and linolenic acid (Abu-Ghazaleh *et al.,* 2002).

Diets rich in linoleic (LA) or linolenic acid (LNA) can increase CLA of milk when dietary oil is accessible to the rumen micro-organisms for biohydrogenation, or to the tissues for endogenous synthesis of CLA (Griinari *et al.,* 2000). When the dietary supply of unsaturated fatty acid is high, or the biohydrogenation process may be incomplete, VA and CLA can escape the rumen and become available for absorption in the lower digestive tract, thus providing substrate (VA) for CLA synthesis in the tissues or CLA for direct absorption (Loor & Herbein, 2003).

Although there are ranges of bacteria that can biohydrogenate FA, *Butyrivibrio fibrisolvens* has been the most extensively investigated. Evidence has also demonstrated the endogenous synthesis of CLA from VA by Δ^9 desaturase enzyme activity, occur primarily in the mammary glands of cows. This also results in the formation of the isomer RA. Endogenous synthesis is the biochemical pathway responsible for the majority of CLA found in products created from milk fat (Kay *et al.,* 2002; Piperova *et al.,* 2002).

2.3.1 Ruminal Biohydrogenation

When polyunsaturated fatty acids (PUFA) in the diet enter the rumen, they are extensively modified through biohydrogenation by rumen micro-organisms with the help of lipases hydrolyzing triglycerides, phospholipids and glycolipids (Khanal & Dhiman, 2004).

Parodi, (1999) established that *cis*-9, *trans*-11 $C_{18:2}$ CLA is the major CLA isomer in milk fat. It was termed rumenic acid (RA), as it is found in such high concentrations in the rumen, and it has been generally assumed that this reflects its escape from complete rumen biohydrogenation. Although isomerization and reduction reactions proceed in a stepwise fashion, different relative amounts of intermediates and products reach the small intestine for absorption. For linoleic acid, the first two steps of the pathway (i.e., biohydrogenation of linoleic acid to RA) happen more rapidly than hydrogenation of VA so that this intermediate (i.e., RA) accumulates in the rumen and is absorbed from the small intestine.

Rumenic acid is produced in the rumen as a stable, first intermediate in the biohydrogenation of dietary LA (*cis*-9, *cis*-12 $C_{18:2}$) by linoleic acid isomerase from the rumen bacteria *Butyrivibrio fibrisolvens* (Harfoot & Hazelworth., 1988; Stanton *et al.,* 1997; Kim *et al.,* 2000)*.* According to Jahreis *et al. (*1999), LA may theoretically be transformed into at least 24 isomers containing conjugated double bonds at positions 7 & 9; 8 & 10; 9 & 11; 10 & 12; 11 & 13; 12 & 14. Each of these isomers may exist in the *cis/cis, cis/trans*, *trans/cis* or

trans/trans configuration. This suggests that several specific isomerases and reductases exist. Changes in the diet often results in bacterial population shifts that alter the pattern of fermentation and products. Almost all isomers of CLA have been identified in food; however, the most commonly occurring CLA in the diet is RA, which is also biologically the most active CLA isomer.

In Figure 2.1 the steps in ruminal biohydrogenation of linoleic and linolenic acid are illustrated.
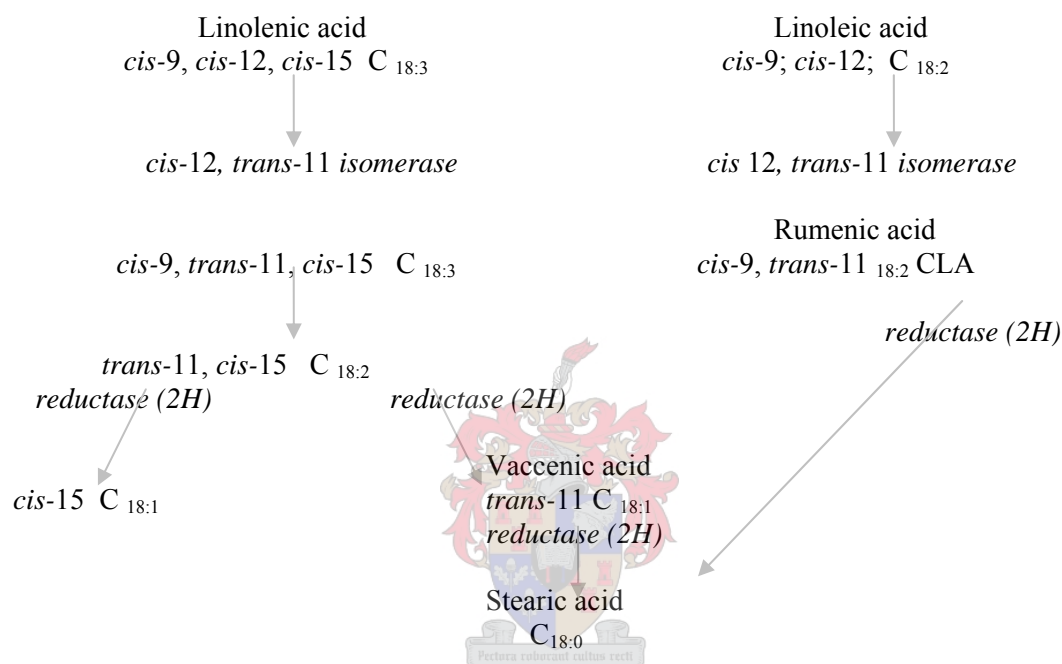
Figure 2.1 Steps for ruminal biohydrogenation of linoleic and linolenic acid from dietary fats. Adapted from Harfoot & Hazelwood (1988), Bauman (2001) and Palmquist (2001).

The first reaction of biohydrogenation for both LA and LNA is isomerization of the *cis*-12 double bond to form a *trans*-11 double bond (figure 2.1). In the case of LA, this produces RA (*cis*-9, *trans*-11 $C_{18:2}$ CLA). Thus a RA isomer is an intermediate in the biohydrogenation of LA, but not LNA (*cis*-9, *cis*-12, *cis*-15 $C_{18:3}$). The next step involves hydrogenation of the *cis*-9 double bond, resulting in VA (*trans*-11 $C_{18:1}$) and *trans*-11, *cis*-15 $C_{18:2}$ for LA and LNA, respectively. An additional step, hydrogenating the *cis*-15 double bond of LNA produces VA, the common intermediate in the biohydrogenation of both FA. The final reaction is hydrogenation of the *trans*-11 double bond to produce stearic acid (Harfoot & Hazelwood, 1998; Kelly *et al*., 1998; Santora *et al*., 2000; Abu-Ghazaleh *et al.,* 2001; Bauman *et al.,* 2001; Piperova *et al*., 2002). If the rumen environment is changed so that biohydrogenation is inhibited, for example, by lowering the pH, more of the intermediate will

escape the rumen and increase the flow of CLA and VA into the duodenum (Piperova *et al.*, 2000; Qiu *et al., 2004a*)

Factors that may affect CLA production in the rumen include the type and source of dietary carbohydrate that may influence the rates of microbial fermentation in a manner that alters the rate of CLA production or utilisation by rumen microbes and ultimately, the concentration of CLA in milk fat. Such an effect could help explain the reported differences in the CLA content of milk fat observed between cows fed fresh forage and cows fed preserved forage. Sugars such as starch, fructosans, pectins, and soluble fiber content, greatly decline during the fermentation process used to preserve forage. The high concentrations of rapidly fermentable starch, sugars and soluble fiber that are found in immature spring pastures may create a rumen environment and conditions that favour a greater production or a reduced utilisation of CLA by rumen bacteria.

Other factors that may affect the rumen environment and microbial population could differ in the grazing animal. For example, passage rate and fluid dilution rate increase because of the high water intake associated with grazing pasture. Meal size, feeding frequency, bite size and time spent ruminating may also differ in cows grazing pasture and these factors may all be important in the alteration of rumen production and utilisation of CLA (Abu-Ghazaleh *et al.*, 2003).

Several studies have shown that CLA occurrs in higher concentrations in milk fat from cows grazing pasture (Parodi, 1997; Palmquist, 2001). The predominant fatty acid in fresh pasture is LNA ($C_{18:3}$, n-3), and CLA is not an intermediate in its biohydrogenation and therefore the high concentrations found in the milk cannot originate only from the rumen (Harfoot & Hazelwood, 1988; Griinary *et al.*, 2000). It is therefore clear that there has to be another place of synthesis. The intermediate from the ruminal biohydrogenation of LNA is VA (*trans*-11 $C_{18:1}$), which according to Palmquist (2001), is the substrate for endogenous synthesis of RA in the tissues.

Griinary & Bauman (1999) hypothesized and established that endogenous synthesis, and not biohydrogenation, is the primary pathway of CLA synthesis. This was later confirmed by Corl *et al.* (2001) and Piperova *et al*. (2002). In 2004, Kay *et al*. concluded that up to 91% of RA is formed through endogenous synthesis in cows on fresh pasture.

2.3.2   Endogenous synthesis

Endogenous synthesis occurs in body tissues and in the lactating cow, primarily in the mammary gland. The catalysing-endogenous synthesis enzyme in the tissues is Δ^9 desaturase and the substrate is VA (*trans*-11 $C_{18:1}$), which is the primary intermediate that escapes complete ruminal biohydrogenation of LA (Chilliard *et al.*, 2000; Griinari *et al.*, 2000; Abu-Ghazaleh *et al.*, 2001; Chouinard *et al.*, 2001; Palmquist, 2001; Piperova *et al.*, 2002).

Endogenous synthesis as described by Bauman *et al.* (2001) involves the absorption of the precursor VA and its subsequent conversion to RA by the Δ^9 desaturase enzyme. For desaturation, FA must first be activated to acyl-CoA synthetase. Desaturased FA has a lower melting point than its more saturated precursors and represents an important determinant of fluidity characteristics of milk fat, depot lipids and cell membranes.

In Figure 2.2 the steps in desaturation of vaccenic acid and endogenous synthesis of RA by Δ^9 desaturase are illustrated.

Vaccenic acid
(*trans*-11 C $_{18:1}$)
+
Coenzyme A

Acyl-CoA synthetase
ATP

Vaccenoyl-CoA +AMP+PPi

Δ^9desaturase
$O_{2;}$ NADP$^+$

Rumenic acid +H_2O
(*cis*-9, *trans*-11 C $_{18:2}$)
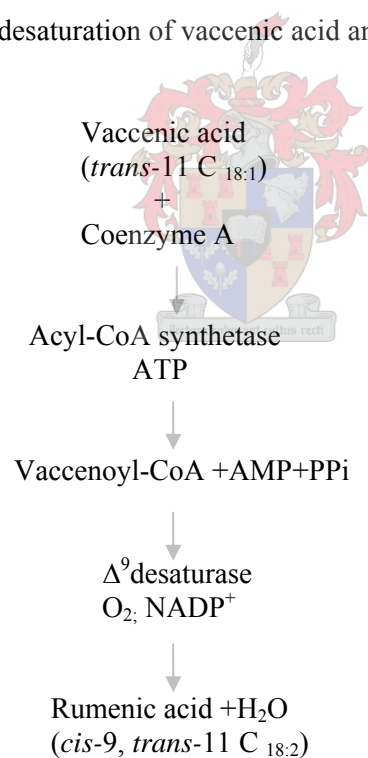
Figure 2.2 Desaturation of VA and endogenous synthesis of RA by Δ^9desaturase. Adapted from Palmquist (2001)

According to Griinari *et al.* (2000), the levels of RA and VA in milk are highly correlated. Thus feeding protocols that increase VA will provide substrate for CLA to be increased by action of Δ^9 desaturase (Kay *et al.*, 2002). Accumulation of VA in the rumen seems to occur

most consistently when the concentration of free $C_{18:2}$ in rumen contents are high. Noble *et al*. (1974), Kepler *et al*. (1996), and Baumgard *et al.* (2000), showed by abomasal infusion of relatively pure CLA isomers, that milk fat synthesis is inhibited by the *trans*-10, *cis*-12 $C_{18:2}$ isomer, but not the *cis*-9, *trans*-11 isomer of $C_{18:2}$. Milk FA of *de nova* origin was decreased to the greatest extent. Though the mechanism of inhibition is not identified, the *trans*-10, *cis*-12 isomer of CLA decreases expression of the Δ^9 desaturase gene. Thus, not only does FA with *trans*-10 isomerization inhibit milk synthesis, they also inhibit endogenous synthesis of CLA by blocking desaturase of VA (Palmquist, 2001).

A linear relationship between the fat content of VA and RA has been observed across a range of diets. This has been generally attributed to their common source as fatty acid intermediates that have escaped complete biohydrogenation in the rumen. However, a linear relationship is also consistent with a precursor – product relationship. A 31 % increase was found with abomasal infusion of VA, which indicates that if there is enough of the precursor for endogenous synthesis, there is a significant increase in milk fat CLA (Bauman *et al.,* 2001; Ward *et al.,* 2002).

2.4   Effects of feeding on CLA synthesis.

This section briefly discusses the effect of animal fats, plant fats, and more specifically fish oil and pasture, on the CLA content of bovine milk fat.

Biohydrogenation of lipids in the rumen is affected by the type and amount of fatty acid substrate, the forage to grain ratio, and the nitrogen content of the diet fed to the ruminant and it is therefore reasonable to assume that the diet of the lactating cow will have a substantial influence on synthesis of CLA (Bauman *et al., 2001*).

Studies suggest that given an adequate dietary intake of LA, dietary constituents that provide ruminal substrate for optimal growth of bacteria producing linoleic acid isomerase will maximise CLA output (Parodi, 1999).

Dietary lipid supplements are often used in formulation of high energy diets for high yielding dairy cows to increase diet energy density, but supplements can also provide substrate for biohydrogenation by rumen bacteria (Chouinard *et al.,* 2001). Supplementing diets with oils from numerous sources can increase the CLA content of milk. Whether the basal diet is a total

mixed ration (TMR) or pasture, the CLA concentration increases with increasing dietary oil content (Parodi, 1999; Dhiman *et al.,* 2000; Chouinard *et al.,* 2001).

The content of CLA in milk fat is dependant on ruminal production of both CLA and *Trans*-11 $C_{18:1}$ and the tissue activity of Δ^9 desaturase and varies widely among dairy herds and between individual animals. This biohydrogenation in turn is dependent on the supply of substrate in the form of PUFA, and in particular dietary LA and LNA (Dhiman *et al*., 1999; Jones *et al*., 2000; Bauman *et al.*, 2001; Kelley *et al*., 2001).

The effect of diet on the fatty acid profile of milk fat is substantial and in a study by Lynch *et al.* (2005), diet was responsible for 95 % of the variance in milk FA. It has been widely researched and confirmed, as shown in Table 2.2 and Table 2.3, that CLA content of cows' milk fat can be increased through nutritional and management practices (Kelley *et al*., 1998a; 2001; Ma *et al*., 1999). Supplying additional fat in the diet, using feeds rich in LA and LNA, or grazing cows on pasture have all been shown to increase the CLA content in milk. The type of fat used, and its processing is important, as it can alter the rumen function and therefore influence the pathways of CLA production. Not only can the addition of fats to the diet of a lactating cow alter the production of CLA, but also more importantly, it can alter the milk yield as well as the milk fat production and influence the DMI of the cow (Palmquist & Beaulieu, 1993; Beaulieu *et al.,* 1995; Dhiman *et al.,* 2000). Even though alterations to the milk fat content are aimed at beneficial components, care also needs to be taken not to increase components that are known to pose a health risk (Offer *et al.,* 1999).

Table 2.2 Fat sources studied for the increase of milk CLA through diet.

Fat source	Milk CLA mg/g fat	MFD	Reference
Pasture	4.78-22.1		Stanton *et al*., 1997; Dhiman *et al*., 1998; Kelly *et al*., 1998b; Collumb *et al*., 2001; Agenas *et al*., 2002; Dewhurst *et al*., 2003; Ward *et al*., 2003
Corn silage	7.3-9	Yes	Dhiman *et al*., 1998
Tallow	1.10	No	Jones *et al*., 2000
Canola oil seeds	2-4.21		Kennely, 1996; Aldrich *et al*., 1997; Ward *et al*., 2002
Cottonseeds	6	Yes	Dhiman *et al*., 1999
Flaxseed	2.16-3.01		Kennely, 1996; Ward *et al*., 2002
Linseed	4.85-7.37		Collumb *et al*., 2004
Rapeseed	5.23 -7		Stanton *et al*., 1997; Lawless *et al*., 1998; Collumb *et al*., 2004
Solin seeds	1.49-13	No	Ward *et al*., 2002;2003
Soybeans	6.9	Yes	Lawless *et al*., 1998; Dhiman *et al*., 1999; 2000
Sunflower seed	7.46-15.46		Collumb *et al*., 2004
Fishmeal	Increased 0.4-3.2 fold	Yes	Abu-Ghazaleh *et al*., 2002
Fish oil	5.2-15.9	Yes	Offer *et al*., 1999; Donovan *et al*., 2000; Jones *et al*., 2000; Baer *et al*., 2001; Chouinard *et al*., 2001; Ahnadi *et al*., 2002; Abu-Ghazaleh *et al*., 2002; Whitlock *et al*., 2002; Abu-Ghazaleh *et al*., 2003
Corn oil	Increased		Griinari *et al*., 1998
Canola oil	2.47 -5.7	No	Ashes *et al*., 2000; Loor & Herbein, 2003
Linseed oil	16.7- 28	No	Kelly *et al*., 1998a; Offer *et al*., 1999
Peanut oil	13	No	Kelly *et al*., 1998a
Soybean oil	6.3-8.4	yes	Piperova *et al*., 2000; Whitlock *et al*., 2002; Loor & Herbein, 2003
Sunflower oil	24.4	No	Kelly *et al*., 1998a
CLA supplement	Linear increase	Yes	Baumgard *et al*., 2002; Bernal-Santos *et al*., 2002 Bell & Kennelly., 2003; Mackle *et al*., 2003

Table 2.3 Calsium salts of fat sources studied for the increase of milk CLA through diet.

Ca salts: CLA	4.92-7.7	Yes	Giesy *et al.*, 2002; De Veth *et al.*, 2005
Ca salts: canola oil	2.3 (mg /g $C_{18:2}$)		Chouinard *et al.*, 2001
Ca salts: Soybean oil	5.4(mg /g $C_{18:2}$)		Chouinard *et al.*, 2001
Ca salts: linseed oil	1.8(mg /g $C_{18:2}$)		Chouinard *et al.*, 2001
Ca salts: palm oil	5.31	No	Perfield *et al.*, 2002

2.4.1 Animal Fats

Supplements are generally composed of animal fat by-products, which contain relatively more saturated FA than found in plant lipid sources. The major fatty acid in animal fat is oleic acid (39-44%) and LA and LNA content of tallow and yellow grease is in the range of 15-17 %, of the total FA. A study by Chouinard *et al.* (2001) evaluated diets with animal fat supplements in different concentrations. Milk yields were similar; however, a shift in milk fatty acid composition did occur with diets containing the fat supplements. Short and medium chain FA as well as palmitic acid were decreased in a linear manner with increasing dietary levels of tallow and yellow grease. In contrast, substantial increases occurred in $C_{18:1}$ and to a lesser extent in $C_{18:0}$. The CLA concentrations of milk fat also increased in a linear manner (p>0.01) with increasing dietary supplements of tallow and yellow grease. However, the magnitude of response was small and milk fat concentrations of CLA were relatively low, compared with those observed with dietary supplements from plant oils.

Linoleic and LNA are of particular importance as ruminal biohydrogenation substrates to produce CLA and *trans*-11 $C_{18:1}$. According to Chouinard *et al.* (2001), these two FA are present at much lower concentrations in animal fat by-products compared to most plant oils.

2.4.2 Fish oils

Fish oils are unique compared to plant oils and animal fats as they contain high concentrations of long chain PUFA. Even though milk fat depression (MFD) is a common problem with the addition of fish oil to diets for lactating dairy cows, it is also of interest because of potential consumer health benefits from enhancing milk concentrations of CLA.

In a study by Chouinard *et al.* (2001), diets containing fish oil had no effect on milk yield, but milk fat content was reduced. In addition, the fish oil supplement altered the fatty acid composition of milk fat, causing a reduction in most saturated FA. This was matched by increases in $C_{18:1}$ and FA grouped as "other". The $C_{18:1}$ is mainly *trans*-11 $C_{18:1}$ and "other"

predominantly represents longer chain PUFA. Similar fish oil effects on milk yield and milk fat composition have been reported (Chilliard & Doreu., 1997; Abu-Ghazaleh et al., 2001; 2002; 2003; Baer et al., 2001; Ahnadi et al., 2002). The CLA concentration of milk fat was substantially increased when cows consumed diets containing fish oil. Others have also observed that dietary supplements with longer chain omega-3 FA resulted in an increase in RA concentration in milk fat, based on results from feeding fishmeal (Dhiman et al., 1999a) and marine algae supplements (Franklin et al., 1999).

In trials by Donovan et al. (2000), it was found that dairy cow diets containing 2 % fish oil, resulted in the highest concentrations of cis-9, trans-11 $C_{18:2}$ in milk and nearly the highest concentrations of $C_{18:3\ n-3}$ FA. Even though DMI and milk fat percentages were decreased with increasing concentrations of fish oil in the diet, milk yield from cows fed 2 % fish oil were similar to milk yields from cows fed control diets. In a trial by Chouinard et al. (2001), fish oil was added at 200 and 400 ml/day to the diet and both levels resulted in a three fold higher RA concentration in the milk fat compared to the control diet.

Feeding lactating dairy cows a blend of fish oil from fishmeal and soybean oil from extruded soybeans resulted in a greater increase in the concentration and yield of milk RA than did the feeding of fishmeal or extruded soybeans separately. According to Dhiman et al. (2005), the fish oil may inhibit bacteria growth or the production of bacterial enzymes that are responsible for the conversion of VA to stearic acid. This creates favourable conditions for endogenous synthesis of CLA from the VA and the $C_{18:2}$ and $C_{18:3}$, provided the oilseeds are then indirectly used for CLA synthesis. Feeding fish oil from fishmeal and extruded soybeans increased milk production. Milk percentage decreased when the blend of fishmeal and extruded soybeans was fed, but milk fat yield was not changed.

Franklin et al. (1999) demonstrated increased quantities of CLA in milk fat of cows supplemented with marine algae. With algae unprotected against ruminal metabolism, the concentrations of RA were increased six to seven fold in milk fat respectively, compared to control diets.

2.4.3   Plant oils

Dietary lipid supplements composed of plant oils are generally not included in ruminant diets (Dhiman et al., 1999). Plant oils can produce inhibitory effects on rumen microbial growth and alter the rumen environment so that a portion of the biohydrogenation process produces

trans-10 $C_{18:1}$ and *trans*-10, *cis*-12 $C_{18:2}$ CLA, two metabolites that are associated with milk fat depression (Dhiman *et al.,* 2000; Chouinard *et al.,* 2001).

Oilseeds, relatively saturated fats and mineral salts of FA are desirable fats for dairy rations because they are rich in $C_{18:2}$ or $C_{18:3}$. If supplemented as crushed seeds or free oil, the FA are accessible to the rumen micro-organisms for biohydrogenation which will lead to an increase in the CLA content of the milk. Addition of polyunsaturated oils in free form can cause milk fat depression (MFD) and should therefore be supplemented in small quantities or in a protected form. It may contain 15-35 % fat which, according to Griinari *et al.* (2000), is highly unsaturated. This may seem undesirable however, the fat within the crushed seed is slowly released into the rumen as the seed is degraded by the ruminal micro-organisms. Therefore, at any point in time, only small amounts of the fats are present in a "free" form in the rumen (Ip *et al.*, 1991; Offer *et al.*, 1999; Donovan *et al.*, 2000; Johnes *et al.,* 2000). An alternative to crushing may be to treat the whole seed with alkaline hydrogen peroxide. Chemical treatment of seeds reduces ruminal biohydrogenation of FA relative to crushed seed and improves postruminal disappearance of FA relative to feeding whole seed (Dhiman *et al.,* 1999a). The alkaline hydrogen treatment of whole seed may be a way of ruminally protecting unsaturated fat, to increase the energy density of diets fed to high producing lactating cows without negatively affecting feed intake, ruminal fermentation, milk production, or milk composition (Aldrich *et al.,* 1997).

As the oil seeds are slowly released during ruminal digestion, it is possible that the accumulation and amount of $C_{18:1}$ trans fatty acid leaving the rumen is reduced, thereby reducing the potential for milk fat depression with raw cracked and roasted cracked oilseeds (Dhiman *et al.,* 2000; Griinari *et al.,* 2000).

Full fat soybeans and cottonseed are commonly used in dairy rations. To make oil more readily available for digestion, the soybeans and cottonseeds may be processed through an extruder to rupture the seeds. Such an increase in rumen substrate in the form of readily available oil from full fat extruded soybeans and full fat cottonseeds, would influence the production of intermediate and end products of biohydrogenation and thus, may alter the fatty acid composition of milk (Dhiman *et al.,* 1999). An increased supply of dietary long-chain FA has been shown to increase the milk fat concentration and inhibit *de novo* synthesis of short and medium-chain FA in the mammary gland. Proportions of long chain unsaturated FA, such as $C_{18:1}$; $C_{18:2}$; CLA and $C_{18:3}$ were found to be higher in the milk of cows fed extruded soybean than those in the milk of cows fed extruded cottonseed diets (Dhiman *et al.*, 1999b; Chouinard *et al.* 2001). However, for the cows fed the extruded cottonseed diet, there

was a higher content of $C_{18:0}$ and a lower unsaturated fatty acid content ($C_{18:1}$; $C_{18:2}$; $C_{18:3}$) in the milk, which suggests more complete rumen biohydrogenation for cows fed a diet of extruded cottonseeds than for cows fed extruded soybeans (Dhiman *et al.,* 1999). This agrees with results by Chouinard *et al.* (2001) using extruded full fat soybeans, cottonseeds and rape seeds. They found that under appropriate dietary conditions, it is possible to maintain normal rumen fermentation while achieving rumen biohydrogenation conditions, which result in a marked increase in CLA content of milk fat.

The same increases in $C_{18:0}$; $C_{18:1}$ and $C_{18:2}$ were found by Lacount *et al.* (1994) with high oil corn grain and high oil corn silage. The high oil corn grain, and to a lesser extent the high oil corn silage, resulted in reductions in the concentration of most short and medium chain FA and palmic acid, with corresponding increases in $C_{18:0}$; $C_{18:1}$; $C_{18:2}$.

Enhancing CLA content of milk through feeding heat-treated soybeans seems to be an economical option. Another option to increase CLA content in milk is to feed soybean or linseed oil. Griinari *et al.* (2000) found that even though feeding free oil decreases milk fat content, the large increase in CLA contents of the milk from cows fed oil, resulted in increased daily yield of CLA per cow. Feeding soybean oil at 2 % of the dietary DM resulted in a 237 % increase in CLA content of milk compared with the control.

Dhiman *et al.* (1999) found that feeding oilseed-supplemented diets significantly decreased the proportion of C_6 to $C_{16:1}$ FA in milk fat compared to control diets. These results agree with studies in the review by Palmquist & Beaulieu (1993) that showed feeding *cis* unsaturated FA in lactation rations lowers milk short chain FA. Lowering concentrations of $C_{14:0}$ and $C_{16:0}$ in milk fat by feeding long chain unsaturated fats may be considered beneficial to human health, due to the association of $C_{14:0}$ and $C_{16:0}$ with hypercholesterolemic affects. Significant elevations in the milk concentrations of $C_{18:2}$; $C_{18:3}$ and $C_{18:1}$ are made possible by feeding solin, flax and canola oilseeds respectively and achieves an increase in the percent of milk fat CLA. There was a positive linear relationship between the proportions of milk fat $C_{18:1}$ *trans*-11 and the proportion of milk fat CLA (Ward *et al.,* 2002).

Chouinard *et al.* (2001) examined effects of different dietary fat supplements and processing methods on CLA. In their first trial, dietary supplements of Ca salts of FA from canola oil, soybean oil and linseed oil increased RA content of milk fat by 3-5 fold over the control diet. In following trials, the effect of processing methods for heat treatment of full fat soybeans was examined. Extrusion, micronizing and roasting resulted in two to three fold higher concentrations of RA in milk fat than the control diet of raw ground soybeans. In another

trial, grain and silage from high oil corn hybrid increased the RA content of milk fat, however, responses were modest with the RA concentrations mg/g fat averaging 4.6 and 2.8 for diets with high oil hybrid and normal hybrid respectively.

Ca salts of plant oils were also used by Bernal-Santos *et al.* (2003) where palm FA and palm FA combined with CLA isomers were evaluated. An increase in CLA with a corresponding reduction in milk fat percentage was noted whilst CLA had no effect on DMI.

Whole canola seed has been explored in the last several years as a fat and protein source for ruminants. It contains high levels of lipid (approximately 55 %) of which over 85 % is C_{18}, with $C_{18:1}$ being the predominant fatty acid (> 60 % of total). Whole canola seed also contains a high amount of protein (20.6 %) with a similar to slightly lower amino acid availability than that of soybean meal (Ashes *et al.,* 1992). The profile of FA in canola seed might cause beneficial changes in milk fat composition, decreased $C_{14:0}$; $C_{16:0}$ and increased $C_{18:1}$ (Ashes *et al.,* 1992), if the unsaturated FA can be protected from ruminal biohydrogenation. However, inclusion of crushed seed should be limited to <4 % of the diet, because of the highly unsaturated fatty acid composition of canola oil, which could negatively effect ruminal fermentation, milk production and milk composition (Donovan *et al.,* 2000).

Ashes *et al.* (1992) used canola seeds protected from ruminal metabolism by emulsification and encapsulation in a matrix of aldahyde – treated protein. They reported that if these treated canola seeds were fed to lactating dairy cows, a 10 % increase in milk fat and no change in milk yield or protein content would result. Feeding the protected canola supplement significantly reduced the proportions of saturated FA $C_{16:0}$, $C_{14:0}$ and $C_{12:0}$ in milk fat, and there were corresponding increases in the proportions of $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$ yield of $C_{18:0}$ monounsaturated and polyunsaturated FA. The latter increased by 54 %, equivalent to 143 g/day (Loor & Herbein, 2003).

According to Loor & Herbein (2003), oil supplementation to the basal diet effectively increased total fatty acid intake by 63%. Feeding canola oil with, or without additional CLA, did not affect total fatty acid intake, but nearly doubled the intakes of *cis*-9 $C_{18:1}$ and $C_{18:n-6}$. Over all, feeding oils increased total concentration of FA in mixed rumen fluid by 69% compared with the control diet (Loor *et al.,* 2002).

2.4.4   Pasture

Fresh herbage is of specific interest as a feedstuff for dairy cows as it is generally a low cost feed and also because of its effects on whole milk composition. Fresh herbage also increases the proportions of C_{18} FA in milk fat, especially the proportions of RA (Agenäs *et al.,* 2002).

Maximum CLA concentrations in milk fat requires optimum ruminal pH and fermentation, which is consistent with the observation that CLA occurs in highest concentrations in milk of pasture fed cows (Ashes *et al.,* 1992; Kelly *et al.,* 1998b; Palmquist, 2001). Low ruminal pH likely alters rumen microbial ecosystem to favour synthesis of the *trans* 10 monoene or conjugated diene, or both. Though pasture consistently increases milk CLA concentration, CLA may also be increased in barn or dry lot feeding systems by supplementing unsaturated oils (Kelly *et al.,* 1998b; Bessa *et al.,* 2000; Palmquist, 2001).

On pasture, there will be marked seasonal variation, with values during the summer period up to three to four times higher than winter values. This is due to the fast growth rate of herbage in the summer months (Banni *et al.,* 1996; Parodi, 1999). Cows have the ability to extract anticarcinogenic components from pasture and feed and transfer them to milk. According to Parodi (1999), it was documented long ago that the CLA content of milk fat is highest in cows grazing lush pasture, assumed to be caused by the high content of PUFA in fresh forage. The CLA content increased almost linearly in milk fat of cows that were provided ⅓ and ⅔ or all of the daily feed allowances from pasture (Dhiman *et al.,* 1999; Palmquist, 2001).

Grazing animals had 5.7 fold higher CLA concentration in milk than cows fed diets containing preserved forage and grain at 50:50 (22.14 vs. 3.9 mg CLA /g FA). In subsequent studies, free oils (rich in LA or LNA) in the diets of dairy cows increased the CLA content of milk (Dhiman *et al.,* 1999).

In a study by Dhiman *et al.* (1999) with different levels of fresh pasture and forage, the CLA content in milk increased linearly as the amount of pasture was increased. Cows grazing pasture alone had 150 and 53 % more CLA in milk fat than cows in the ⅓, ⅔ pasture treatments, respectively, and 500 % more CLA in milk fat than cows fed diets containing forage and grain in a 50:50 ratio. Calculated forage and grain ratios in ⅓ pasture, ⅔ and all pasture treatments were 46:54; 80:20 and 100:0 respectively. The proportion of $C_{18:3}$ increased in milk fat as the amount of feed from pasture increased in the diet. Cows grazing permanent natural pasture had 500% more CLA compared with cows feed TMR containing

conserved forage and grain in a 50:50 ratio. Feeding pasture grass in dry form as hay did not influence milk CLA content (Dhiman *et al.,* 1999).

2.4.5   Copper

Copper is involved in uptake of iron from the diet, in its incorporation into haemoglobin and in the oxygen metabolism in the red blood cells as well as being involved in many other enzyme systems, and now it also has an effect on CLA synthesis. Copper deficiency can be either primary, where there is an absolute lack of copper in the soil and hence the pasture or secondary, when there is adequate copper in the soil but other causes of copper being unavailable to the cow (e.g. high molybdenum).

It has been shown that a lack of copper in the diet of dairy cows increases CLA synthesis. Copper depletion decreases copper in the milk and the milk concentration of copper may be a more accurate indicator of the animal's copper status than plasma levels of copper. It is thought that the copper depletion inhibits biohydrogenation in the rumen and therefore increases CLA in milk through incomplete biohydrogenation of LA (Molares *et al.,* 2000a, b).

Copper acts as a pro-oxidant in milk and with increased unsaturation in milk fat, it can accelerate the development of off-flavours in milk products. According to Thompson *et al*. (1973), copper not only influences biohydrogenation in the rumen, but can also effect endogenous synthesis of CLA by influencing the activity of stearoyl-CoA desaturase. Jerseys have a lower activity of stearoyl-CoA desaturase than Holsteins and it is documented that they absorb copper better than Holsteins (Beaulieu & Palmquist, 1995). It is therefore possible that copper can have an influence on the breed effect found in CLA synthesis.

2.5   CLA variation in milk

It has been widely reported that a considerable amount of variation in CLA content of milk fat exists (Bauman *et al.*, 2001; Dhiman *et al.*, 2005). This variation can be attributed to several factors.

Seasonal variation has a substantial influence (Lock & Garnsworthy, 2003), which can be ascribed to changes in herbage composition on pastures, maturity of grasses, and a change in concentrates being fed. Management systems will also have an effect on CLA content of the herd which can be due to different diets, as shown in Table 2.5, and feeding practices used in different systems (Jahreis *et al.*, 1996; Dhiman *et al.*, 2005). Slight differences due to animal breed, as shown in Table 2.4, have also been reported (Lawless *et al.*, 1999; Whitlock *et al.*, 2002; Dhiman *et al.*, 2005).

These factors would influence the whole herd, but large variation has also been reported between individual animals in a herd (Kelly *et al.*, 1998a, b; Peterson *et al.*, 2002). It is also reported that the cows will normally rank in the same order within the herd even if the diet is changed (Lawless *et al.*, 1999; Peterson *et al.*, 2002)

The first factor that would cause variation in individual animals is the population of micro-organisms in the rumen of the animal. There are several factors that effect this population, as the micro-organisms are very sensitive to rumen pH (Martin & Jenkins. 2002; Qiu *et al.*, 2004a), lipid substrates in the diet (Qiu *et al.*, 2004a; b), forage to grain ratio (Qiu *et al.*, 2004a), passage rate and fluid dilution. Meals size, feeding frequency, bite size and time spent ruminating also differs between cows. Another factor that would cause a variation among individual animals is gene expression and activity of the Δ^9desaturase enzyme (Kelly *et al.,* 1998; Bauman *et al.*, 2001; Palmquist, 2001). A difference in CLA content of milk fat has also been reported from cows in different stages of lactation with cows with more than 7 lactations having more CLA in milk fat than cows having 1-3 lactations (Palmquist & Beaulieu, 1992; Dhiman *et al.*, 2005).

In a study by Lock *et al.* (2005), DMI, milk yield, milk fat content and milk fat yield were investigated. They concluded that these parameters had no significant effect (R^2 values all < 0.08) on RA content of milk fat and do not need to be considered or included in management practices aimed at increasing RA content of milk fat.

Table 2.4 Dietary factors that have an effect on CLA synthesis.

Dietary factor	Effect on the content of CLA in milk fat
Lipid substrate	
Unsaturated vs saturated fat	Increased by addition of saturated fat
Plant oils	
Type of plant oil	Variable increase
Level of plant oil	Non-linear dose dependant increase
Ca salts of plant oils	Increase
High oil plant seeds	
Raw seeds	No effect
Processed seeds	Variable effect
High oil corn and grain silage	Minimal effect
Animal fat by products	Minimal effect
Modifiers of rumen biohydrogenation	
Forage to concentrate ratio	Variable effect
NSC[1] level	Minimal effect
Restricted feeding	Variable effect
Fish oils	Increase
Fish meal	Minimal effect
Marine algae	Increase
Ionophores	Variable effect
Dietary buffers	Minimal effect
Combination	
Pasture	Higher than on conserved forages
Plant oil + high NSC[1] diet	Maximal effect but transient
Processed seeds + fish oil	Additive effect
Growth stage of forage	Increased with less mature forage
Dietary supplements	
CLA supplements	Linear dose dependant increase
trans-11 $C_{18:1}$ supplements	Non-linear, dose dependant increase

[1]non-structural carbohydrates

Table 2.5 CLA synthesis by different breeds. Information taken from Dhiman *et al*. (2005)

Breed	Diet type	Diet	CLA content of milk fat
Montbeliard	Pasture	A	1.85 %
Holstein- Friesian	Pasture	A	1.66 %
Normande cows	Pasture	A	1.64 %
Holstein- Friesian	Pasture	B	0.57 %
Jersey	Pasture	B	0.47 %
Holstein- Friesian	Conserved forages	C	> Jersey
Jersey	Conserved forages	C	< Holstein- Friesian
Brown Swiss	n/a	D	> Holstein- Friesian
Holstein- Friesian	n/a	D	< Brown Swiss
Brown Swiss	Conserved forages and grain	E	0.41 %
Holstein- Friesian	Conserved forages and grain	E	0.44 %
Ayrshire	Conserved forages and grain	F	0.68 %
Guernsey	Conserved forages and grain	F	0.34 %
Jersey	Conserved forages and grain	F	0.34 %

2.6   CLA and milk fat depression.

Milk fat depression (MFD) is a well known occurrence, especially where diets are being supplemented with fats. With the supplementation of diets with CLA, a definite relationship was found between MFD and CLA (Griinari & Bauman, 1999; Baumgard *et al*., 2000; 2001: Bauman *et al*., 2001). Even though, economically speaking, MFD should be avoided on commercial dairy farms, there are some scenarios where this MFD can hold an advantage for the cow.

During severe heat stress or poor weather conditions animals cannot consume enough feed to meet energy requirments. Another example is at peak production when cows produce more milk than energy intake can cater for. Researchers used this natural occurence of MFD to combat the negative energy balance at the onset of milk production by supplementing the diet with CLA in early lactation (Bauman *et al.,* 2001 Perfield *et al.,* 2002). However, Bernal – Santos *et al.* (2003) found that if CLA is fed two weeks prepartum and in early lactation the cow seems to respond to the MFD by directing more energy to the synthesis of milk instead of improving the energy balance.

Thanks to improved analytical procedures, scientists could establish that MFD was related to an increase in not just *trans* C $_{18:1}$, which would include VA, but specifically to an increase in *trans*-10 C $_{18:1}$ (Griinari *et al*., 1998). The *trans*-10, *cis*–12 C$_{18:2}$ is an intermediate in the formation of trans-10 C $_{18:1}$ from the biohydrogenation of linoleic and linolenic acid. It was shown by Griinari *et al*. (1999a) that there is a linear relationship between *trans*-10, *cis*-12 C$_{18:2}$ CLA and *trans*-10 C$_{18:1}$. They also identified a curvilinear relationship between the increases in milk fat content of *trans*-10, *cis*-12 C$_{18:2}$ CLA and the reduction of milk fat yield in cows fed different MFD diets. This was confirmed by Baumgard *et al*. (2001) and Palmquist (2001) when they established that *trans*-10, *cis*-12 C$_{18:2}$ CLA and not RA is responsible for MFD. In 2003 Perfield *et al*. (2004) confirmed that *trans*-10, *cis*-12 C $_{18:2}$ is responsible for MFD and that *trans*-8, *cis*-10 C$_{18:2;}$ *cis*-11, *trans*-13 C$_{18:2;}$ and *cis*-9, *trans*-11 C$_{18:2}$ had no effect on milk fat percentage.

In Figure 2.3 the recognized pathways of ruminal biohydrogenation of linoleic and linolenic acids involving *trans*-10, *cis*-12 isomerases is illustrated.
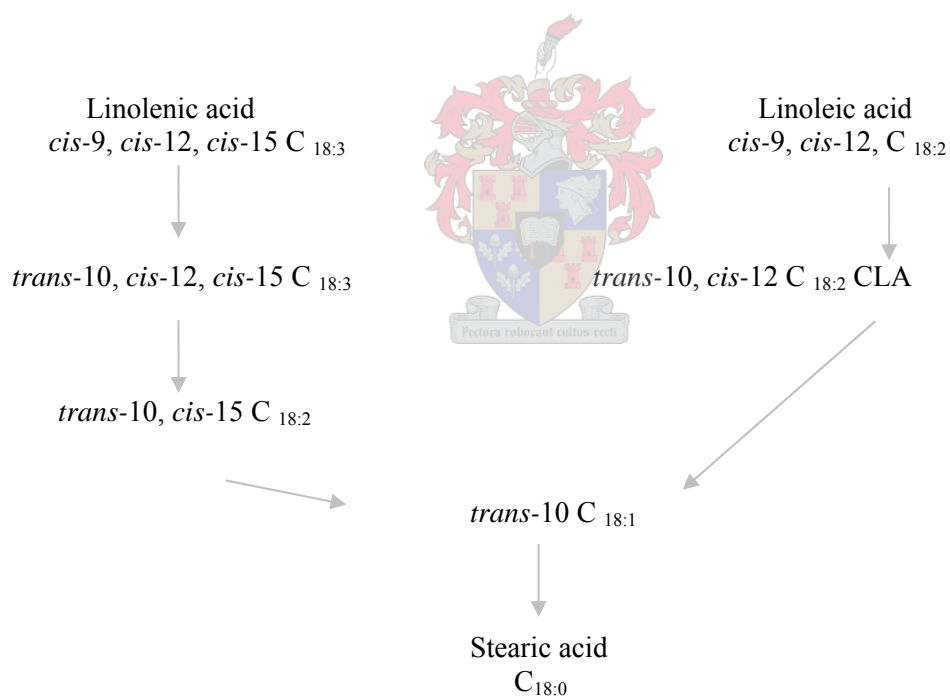
Figure 2.3 Putative pathways of ruminal biohydrogenation of linoleic and linolenic acids involving *trans*-10, *cis*-12 isomerases. Adapted from Bauman *et al*. (2001).

2.7   Stability of CLA in milk

Evaluating the stability of CLA enriched milk, as it is presented to the consumer, has established that the CLA content of products are relatively stable during processing, manufacturing and storage conditions that are typical for the dairy food industry (Bauman *et al.,* 2001; Campbell *et al.*, 2003). The only account where processing negatively influenced the CLA, or more specific the RA content and also the *trans* FA of the milk fat, was with microwave heating of cheese. During heating of cheese in the microwave oven for five minutes, it was found that *trans* FA were increased up to 31 % and CLA content significantly decreased by 21 %. Heating the cheese for 10 minutes in the microwave oven caused a reduction of 53 % of CLA compared to conventional heating (Herzallah *et al.,* 2005).

Even though some research has been conducted on the antioxidant properties of CLA, conflicting results were obtained from these studies (van den Berg *et al.,* 1995; and Yu *et al.,* 2002).

Recently milk that was naturally enhanced with RA and VA was subjected to evaluation to determine the milks' susceptibility to oxidized off-flavours. Milk with a 2 % fat content was pasteurized, homogenized and exposed to light. No difference was found between the CLA enhanced milk and the control over a 14 day period. No flavour differences were detected over the shelf life of the product, and no oxidized off-flavours were detected during sensory evaluation. With this Lynch *et al.* (2005) concluded that it is possible to naturally increase the RA and VA content of milk fat with up to 7.5- and 8-fold respectively and maintain acceptable sensory characteristics. This supports the findings of Baer *et al.* (2001) and Kitessa *et al.* (2003) who found that milk or butter from cows on a diet supplemented with fish oil had comparable sensory characteristics than that of control groups. Baer *et al.* (2001) also found that peroxide values from CLA enriched butter were similar to that of the control group.

2.8   Conclusions

With the potent anticarcinogenic and other health benefits that can be derived from consuming conjugated linoleic acids, it should be a major goal for researchers and producers to find feasible ways of increasing the CLA in milk fat.

Dietary manipulation of CLA for lactating dairy cows by supplementing with fats, seem to be an effective means to provide a dietary strategy not only to offset the negative body energy balance that occurs in early lactation, but to also increase the production of CLA. Over all, several dietary manipulations involving lipid sources and processing methods have been identified that allow for a marked increase in the conjugated linoleic acid content of milk. It seems to be an economical option to make use of full fat seeds that are processed so that a portion of their unsaturated fatty acids becomes available in the rumen for microbial biohydrogenation while avoiding less desirable effects on ruminal bacteria.

Dietary addition of plant oils and fish oil results in substantial increases in milk fat cis-9, *trans*-11 CLA concentrations. This is possible without affecting milk and milk fat yield, but by changing the milk fat content. With the increase in milk concentrations of $C_{18:1;}$ $C_{18:2;}$ $C_{18:3}$ there is a reduction in the concentrations of $C_{14:0}$ and $C_{16:0}$. However, feeding greater amounts of fish oil than 2 % of the diet increases milk CLA concentrations by 3.2 fold but decreases both milk fat percentages and yield.

Due to the large difference in CLA content in season and management practices, it will be difficult to maintain a certain level of CLA throughout the year.

With the difference in CLA synthesis in individual animals, it should be possible to select for higher CLA synthesis in the long run.

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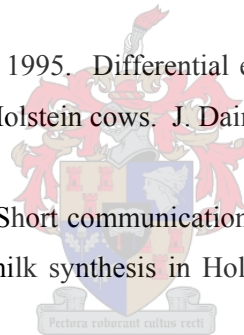
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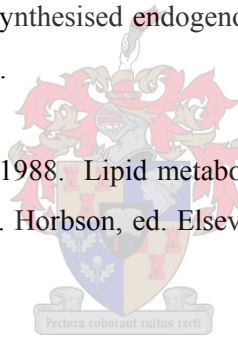
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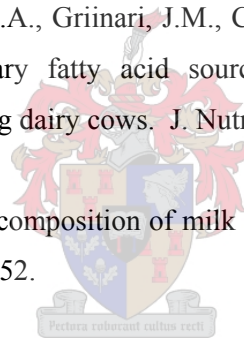
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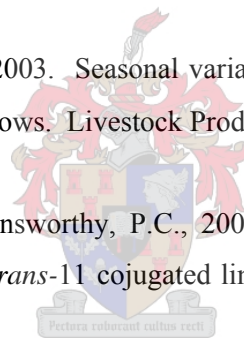
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CHAPTER 3

General materials and methods

3.1 First trial

The objective of this study was to get an indication of the average CLA content of bovine milk from production systems that are commonly used in the Western Cape Province, South Africa. This study was therefore conducted to provide a reference point for CLA concentration in milk bulk tanks from herds that are either pasture based or on a total mixed ration (TMR) system.

3.1.1   Experimental design, treatments and data collection.

Milk samples were collected in February and March 2003 from bulk tanks from 24 dairy herds and three silos of dairy co-operatives to determine the CLA status of milk in the Western Cape.

The bulk tank samples were collected from six TMR based Holstein and six TMR based Jersey herds, as well as from six pasture based Holstein and six pasture based Jersey herds. In the TMR systems diets consisted of typical high concentrate mixtures, while in the pasture based herds, pastures consisted predominantly of lush ryegrass with varying levels of kikuyu or clover.

Silo samples were from a mixed herd silo and a Jersey herd silo from TMR based herds in the Stellenbosch area and from a mixed herd silo from pasture based herds in the Mosselbay area.

Each milk sample of 250 ml was divided into 2 sub samples, one for the determination of protein, fat and lactose, and the other for the determination of fatty acid composition. Samples were kept below 5° C and transported to the laboratory the day after collection. The samples for milk composition were preserved at collection with bronopol-B2 preservative and analyzed with the aid of a Milk- O Scan 133B at the ANPI Dairy Lab at Elsenburg, Stellenbosch. Milk samples for fatty acid analysis were freeze dried and vacuum-sealed at the department of Animal Science at the Stellenbosch University, and then sent to the Skaggs Nutrition Laboratory at Utah State University, USA, for quantifying fatty acids (FA) using gas chromatography.

Data were subjected to a one way ANOVA with the aid of the GLM procedure of SAS 8.02 (2000). Least square means were determined and a Bonferoni test was used to separate means. Significance was declared at $P \leq 0.05$.

3.2 Second trial

This pasture based feeding study was conducted at the Outiniqua Experimental Farm near George, South Africa, in the last quarter of 2004. The CLA trial was done simultaneously with a commercial Flavomycin trial using the same control group. There were therefore three groups of 20 cows kept as one herd. In this thesis the author will only report on data from the CLA and control group, but in the layout of the trial the author will discuss how the whole herd was treated.

3.2.1   Experimental design, treatments and data collection.

Sixty multiparous Jersey cows in different stages of lactation were assigned to the control group, the Flavomycin treatment group or the CLA treatment group. Groups were balanced for days in milk (DIM), milk production and lactation number to obtain homogenous groups. The milk production criterium was based on the average of a three-week production period, two weeks before the beginning of the trial. The average milk production, DIM and lactation number of cows in treatment groups at the onset of the trial is given in Table 3.1 and the separation of lactation number in treatment groups is given in Table 3.2.

Table 3.1 Average milk production, DIM and lactation number of cows in treatment groups at the onset of the trial.

	Average milk production over the preceding 3 weeks	Average DIM 20 August 2004	Range DIM	Average Lactation number
Control	21.57	77.35	(25-157)	4.40
Flavomycin	21.49	79.10	(22-130)	4.35
CLA	21.51	78.80	(25-122)	4.50

Table 3.2 Separation of lactation number in treatment groups.

Lactation number	Control	Flavomycin	CLA
10	1	0	0
9	0	1	2
8	1	1	0
7	1	1	1
6	2	2	2
5	1	1	2
4	6	6	6
3	6	6	5
2	2	2	2
1	0	0	0
Average	4.40	4.35	4.50
Count	20	20	20

The experimental period was 12 weeks; the first two weeks of the experiment served as a pre-trial period that allowed the cows to adapt to the new diets. Measurements were made during weeks 4, 6, 8, 10 and 12. Milk yield of each cow was recorded electronically twice daily. Afternoon, 15:00, and morning, 05:00 milk samples were collected at a rate of 10 ml /L of milk produced, every two weeks on weeks 4, 6, 8, 10 and 12. Part of the milk sample, 40ml, was preserved with bronopol-B2 preservative for milk composition analysis, and the other part, 400ml, was frozen at -20 °C and then freeze-dried and vacuum sealed for fatty acid analysis at the Skaggs Nutrition Laboratory, Utah State University, USA. The samples were analyzed for fat, protein, lactose and milk urea nitrogen by the TAURUS milk analysis laboratory, Irene. Final milk composition was expressed based on weighted a.m. and p.m. milk yields.

Every two weeks the cows were weighed and the body condition was scored on a scale of 0 - 5. The scale was adapted to measure smaller differences between cows in such a manner that a cow in a better condition than a 3, but not good enough for a 3.5 would get a 3+, in the same way a cow that was not a 3; but better than a 2.5 would get a 3-. To be able to convert that in numeric terms, the + or - was indicated in increments of 0.25. The cow that was given a 3+ would then be 3.25 and the cow with a 3- would be 2.75.

To ensure an accurate body mass, cows were weighed on two consecutive days at the start of the afternoon milking and the BCS was always done by the same experienced stockman.

3.2.2   Concentrate supplement

Concentrate samples were collected weekly and analyzed for dry matter (DM), crude fat (CF), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) copper (Cu) and ash.   Analytical methods are described in section 3-4 under methodology. The mean composition of the concentrate supplement is given in Table 3.3.

Table 3.3 Mean calculated composition of the concentrate supplement that was provided to cows on pasture.

Average composition	
DM %	89
Fat %	4
Prot %	11
NDF %	9
ADF %	4
Ash %	5
Cu mg/kg	30.6
Energy (calculated)	15 MJ ME/kg (DM)

3.2.3   Pasture samples

Pasture samples from each week during the trial period were analyzed for DM, CP, CF, NDF, ADF, Cu, and ash and the results thereof are given in Table 3.4.

Table 3.4 Chemical composition of the pasture over weeks 2, 3, 4, 5, 7, 8, 9 and 10.

Week	DM %	Crude Fat % of DM	Crude Protein % of DM	NDF % of DM	ADF % of DM	Ash % of DM	Copper mg/kg of DM
2	11.7	4	23.7	47.2	29	11.9	5.6
3	11.5	4	26.7	42.8	27	13.3	7.2
4	11.2	4	20.2	37.4	25	8.6	6.8
5	16.9	4	23.0	45.9	27	11.9	5.7
7	12.5	4	27.5	52.1	28	12.7	6.1
8	14.0	4	23.7	49.8	26	11.3	6.5
9	18.3	3	22.2	51.0	27	11.3	5.7
10	21.3	3	20.7	52.8	28	9.4	6.5

3.2.4   Management and housing of cows

The three trial groups were housed together as one herd to minimize environmental differences. The cows were tagged with color-coded tags to ease identification. The control group was given red tags, the flavomycin group white- and the CLA group blue tags. Before each milking the three treatment groups were separated, and each group of 20 cows were milked and fed separately. After being milked, the groups were combined and returned to the pasture for grazing.

In the first week of the trial, cow Number 1 was hurt and replaced by another cow that was used for the remainder of the trial. Therefore there is no body weight or BCS value for this cow for the first week of the pre-trial period. In the 6th week of the trial cow Number 44 contracted severe eczema of the udder. She was kept in the group to keep the size of the group, but she was not milked for trial purposes and is thus missing data for the trial, leaving the CLA croup with a count of 19 cows.

3.2.5   Feeding program

The herd had 24 hour access to pasture and each cow received 6 kg of a high energy concentrate per day (Table 3.3). Fresh water was available at all times during grazing.
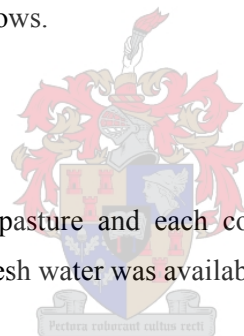
The pasture consisted mostly of kikuyu that was over sown with ryegrass during the winter. At the start of the trial in September, the herbage consisted predominantly of ryegrass, but as temperatures increased during spring, the kikuyu started growing and contributed more towards the herbage. When the trial ended in December, the pasture was still predominantly ryegrass, but with a considerable amount of kikuyu.

As the levels of milk production increase, the need for more concentrated sources of energy and protein to supplement that of the grass becomes necessary (Van der Merwe & Smith, 1977). Energy deficiency, either because of limitations in the feed, or due to limitation in DM intake (DMI), is one of the major reasons for underperformance by an animal. Protein, after DMI and energy, is the third most important limiting factor in dairy cow nutrition. To overcome these limitations concentrates normally contain a higher energy and protein density than forages and can therefore be supplemented in small quantities.

The concentrate mixture was balanced for minerals and vitamins, and was formulated to meet the nutrient demand of lactating cows according to the NRC recommendations. The Cornell Net Carbohydrate and Protein System (CNCPS) were used in formulating the concentrate mixture.

The concentrate mixture was split into two equal portions and fed during morning, 05:00, and afternoon, 15:00, milking in an individual feeding system. The Flavomycin treatment group received a total of 6.25 g of Flavomycin per day and the CLA treatment group received 70 g of commercial CLA (Luta CLA 20 P) per day. It was given with their concentrate, administered as a top-dressing and hand mixed into the concentrates just before feeding. The Flavomycin and CLA were thus also given in two separate portions.

3.2.6   Pasture management and allocation of daily grazing

Grass and other forage crops are, and will remain, the foundation of any dairy cow diet. Although popular, forages are insufficient for high-yielding milking cows and usually require supplementing with feeds rich in energy, protein, or other nutrients (Heard *et al.,* 2004). However, feeding supplements to grazing dairy cows will have a substitution effect on the herbage intake, as cows generally substitute the supplement for some of the pasture they would otherwise have eaten. Voluntary dry matter intake is most often the factor that limits production from pasture.

Grazed pasture is the cheapest feed for the dairy cow, so it is important that its use is maximized as far as possible. Two types of growth occur in grasses: vegetative growth and reproductive growth. Vegetative growth, otherwise known as tillering, consists of new shoots developing at ground level, which ultimately become new plants. Each mother plant can have up to 25 single stem units called tillers. Each tiller has its own roots and leaves and ultimately becomes a new plant. If this tiller stops producing new leaves the pasture stopped growing. To prevent this, frequent defoliation needs to take place. Tillering occurs mainly in autumn and winter when growth is slow, and is stimulated by defoliation at that time of year. Defoliation can either be by grazing or by cutting.

Reproductive growth involves stem elongation and the development of the flowering head; it occurs in spring and summer and is associated with little or no tillering of the plant. It follows that allowing the plant to enter reproductive growth repeatedly is likely to result in an open sward with fewer tillers than in a sward, where stem development is interrupted or prevented by frequent cutting or grazing. When the tiller has tree-leaves, it is called the tree-

leaf stage of re-growth. If it is grazed at this time, the tiller stops root growth and utilizes its energy to re-grow leaves. If this does not happen the plant will have no leaves for photosynthesis and will die. As soon as the tiller has one mature leaf, it will start to store energy and food reserves again and more leaves will develop. When the third leave develops a growth point, the plant is once again at the three-leaf stage and ready for grazing. The re-growth rate will depend on ground temperature and the size of the leaf depends on availability of water and nutrients but normally re-growth ranges between 3 - 6 weeks.

For re-growth to take place the growth point of the leaves need sunlight. If this is not attained, either because the plant is not grazed, or because of under grazing and the growth point is over shadowed, re-growth will stop and the plant will die (Botha & Oberholzer, 1997).

The most productive and most persistent grass swards are therefore those that are grazed hard in autumn to stimulate tillering, and those that are grazed intensively in spring to delay reproductive growth. The key to increase plant density is therefore intensive grazing in autumn or spring to stimulate tillering (Wolfson & Tainton, 2000).

The key to success in grazing is controlling daily herbage allowance per cow, to make sure the plants are grazed short enough to prevent over shadowing and to stimulate re-growth. This requires a flexible approach to stocking rate or pasture camps, since herbage growth fluctuates.
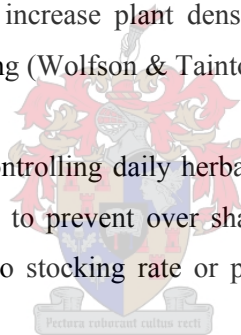
The pasture used in this trial was divided in narrow strips of 15 m width and the length was divided into plots of 15 m by placing sprinklers every 15 m over the length of the strip. Therefore every plot was 15 m x 15 m = 225 m^2. There were enough strips with 225 m^2 plots to ensure growth time of approximately 3 - 4 weeks in spring for the forage to re-grow after being grazed. This made the allocation of the pasture easy, because during different growth rates the yield (kg DM/ha) could easily be calculated and one could determine how many plots or sprinklers in an ungrazed strip should be allocated at a time, to be sufficient for the size of the group. The rest of the plots were closed off with the use of an electric fence. The animals were also allowed to access a recent grazed plot. This gave them a resting area, so that animals spent less time on the newly allocated pasture, therefore minimizing fouling and trampling on the ungrazed strip. No plots were included within the grazed area for more than a day, to limit the period that any of the pastures were exposed to grazing, trampling and fouling.

Strip grazing requires more labour than fixed camp systems, but is better manageable and ensures that the pasture is evenly and efficiently utilized.

The pastures were allocated daily according to results of weekly regressions to ensure that there was sufficient food for all the cows and that the pastures were utilized to a maximum. Pasture measurements were taken daily before and after grazing, using a rising plate pasture meter (Botha, 2003; Stockdale *et al.*, 2003). Dietary allowances of pastures were adjusted weekly, when necessary, to account for small changes in dry matter (DM) content and yield per hectare.

The rising plate pasture meter technique used to determine the DM yield, as well as DM intake, is a simple method, which gives reasonably accurate measurements. The plate of the rising plate pasture meter is placed horizontally on the pasture. The rod connected to the measuring mechanism rests on the soil, giving the distance from the soil to the top of the plants where the plate is resting. This mechanism is very easy to use and gives the operator the opportunity to make up to 100 measurements in as little as 5 min. The high volume of measurements increases the accuracy of the regression, especially if there is a large variation of plant sward height on the pasture.

To calculate a regression line for the specific pasture, nine pasture samples were taken every week. A metal ring with a surface area of 0.0999 m^2 (ø = 357 mm) was used to select three high, three medium and three low sward-heights pasture samples. The samples were cut using hand shears. All the plant material inside the ring was cut down to 3 cm above the ground, bagged and marked. The samples were weighed, dried at 60 °C for 48 hours and then weighed again to obtain the dry matter percentage. This allowed meter, or sward height, readings to be converted to herbage mass.

Y = DM Yield (Kg DM /Ha)

Sg = dried pasture sample in grams

Am^2 = 0.0999 m^2 (surface area of the ring)

1 kg = 1000 g

1 Ha = 10000 m^2 / ha

$$Y = \frac{Sg \times 1kg \times 10000m^2/ha}{Am^2 \times 1000g}$$

The yield values were plotted on a graph and a regression line was fitted to the plots. The equation of the regression line was y = mx + c.

y	=	the yield
m	=	the gradient of the regression line
x	=	the height of the pasture
c	=	the correction factor.

To obtain the yield per Ha, 100 measurements were taken with the rising plate pasture meter. The average was then used as x in the equation and the yield per Ha was calculated. Hundred measurements were taken before grazing, and 100 after grazing and the difference in height was then used in the equation to calculate intake.

The amount of pasture allocated per cow per day was calculated as follows:

BW	=	Average body weight of jersey cow; 400 kg
N	=	1.3 % of body weight is taken in as NDF (1.2 % ± 0.1; NRC, 1988)
If	=	kg of NDF requirement
F	=	NDF content of pasture (DM). = 40%
I	=	DM intake per cow per day if grazing only
S	=	Substitution rate of 0.093 for concentrates (Faverdine, 1991)
SC	=	Amount of substitution per kg of concentrates
C	=	Kg of concentrates offered
Is	=	DM intake with concentrate substitution

If	=	BW x N
	=	400 kg x 1.3 NDF/100
	=	5.2 kg NDF

I	=	If x F
	=	5.2 kg NDF x 100 kg DM/40 kg NDF
	=	13 kg DM

SC	=	S x C
	=	0.093 x 6 kg
	=	0.558 kg

$$\begin{aligned}
\text{Is} \quad &= \quad \text{I} - (\text{SC} \times \text{C}) \\
&= \quad 13 \text{ kg DM} - (0.558 \text{ kg} \times 6 \text{ kg}) \\
&= \quad 13 \text{ kg DM} - 3.348 \text{ kg DM} \\
&= \quad 9.652 \text{ kg DM}
\end{aligned}$$

To ensure that the cows were given sufficient pasture to graze, 10 kg of DM per cow per day were allocated. For 60 cows, the allocation was thus 600 kg per day (300 kg per feeding).

From the equation of the regression line the yield per ha could be calculated. To calculate how many sprinklers should be allocated for a single feeding (300 kg DM for 60 cows) when the pasture has a yield of **y, y** = mx + c, kg DM / ha the following equation was used:

300 kg DM x (1ha/**y** kg DM/ha) x (10000 m^2/1 ha) x (1 sprinkler/225 m^2)
= 13333.3/**y** sprinklers

With the use of the rising plate pasture meter and using pasture plots of known size, it is easy to manage pastures properly. By also calculating weekly regressions, pastures can be allocated accurately (as the DM contents of the pasture changes over time) thereby ensuring proper grazing of the pasture with as little as possible going to waste.

In Figure 3.1 to 3.3 the regression lines for a week in September, October and November, respectively, are shown. From the gradient on the regression lines it can be seen how the DM yield changed over the weeks.
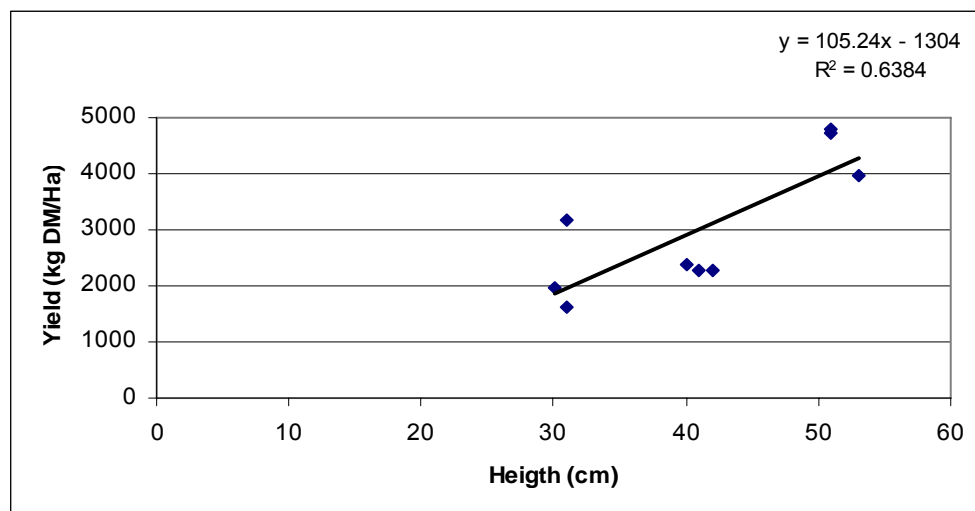
Figure 3.1 Regression results on pasture for the week of September the 20th.
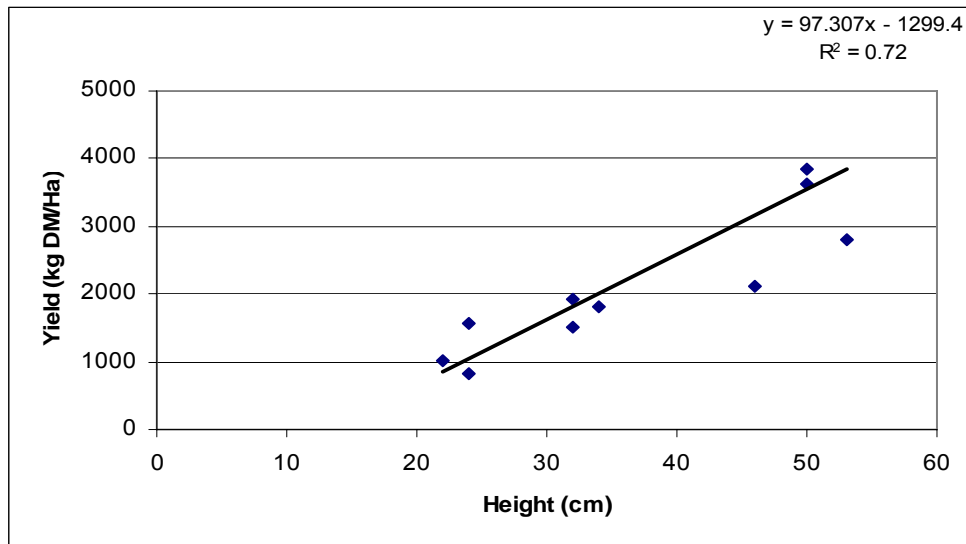
Figure 3.2 Regression results on pasture for the week of October 18th.
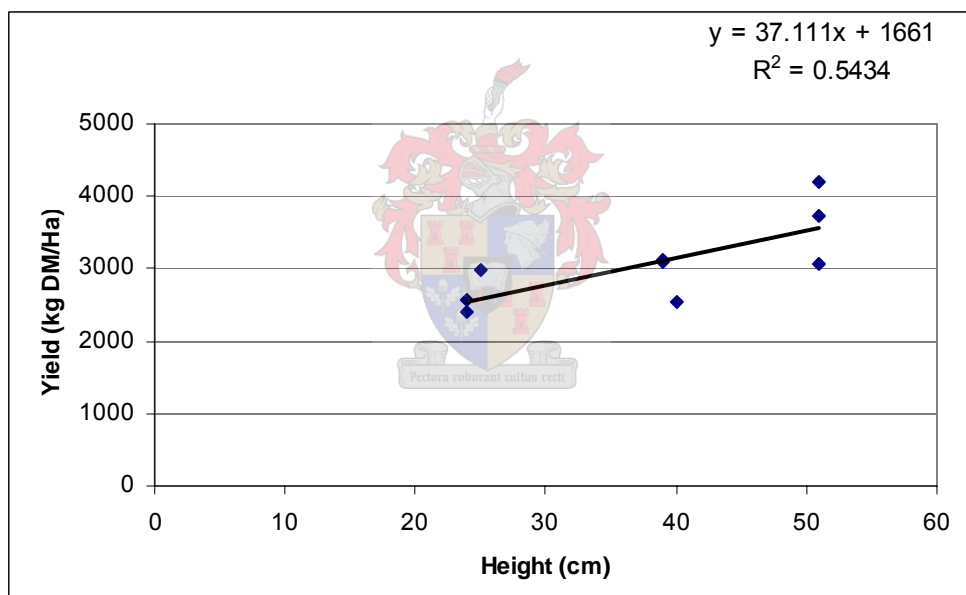
Figure 3.3 Regression results on pasture for the week of November 26th.

3.2.7   Statistical analysis

Cows were stratified according to milk production, days in milk (DIM) and lactation number and then randomly divided into three groups of 20. This resulted in three comparative groups.

For each data collection period the data was subjected to a one way analysis of variance (ANOVA) using STATISTICA 7. Where appropriate, an ANOVA of repeated measures were done. Means were separated with a Bonferoni test. Significans was declared at $P \leq 0.05$.

3.3 Third trial

This study was conducted at the Welgevallen Experimental Farm of the Stellenbosch University. This study was from end September to mid December 2004 and 12 high yielding multiparous Holstein cows in mid lactation were used.

3.3.1 Experimental design, treatments and data collection.

The trial was designed as a 4 x 4 Latin square (n=3). The four groups (3 cows per group) were fed four diets, one treatment diet per group in the first period. After completion of the first period, the groups were given a different treatment until each group received each diet over the four periods. Each period was planned to last 18 days, but in the 3rd period some of the cows fell ill and milk production decreased drastically. The period was extended by six days to give the cows the opportunity to regain their normal milk production before samples were taken. The last period had to be shortened by four days, because of limited feed supply.

Cows were milked twice daily at 5:30 and again at 16:00. Yields were recorded by hand at each milking. Milk samples were collected, 10 ml/l of milk produced, on the evening of day 17, and morning of day 18. The two milk samples from each cow were pooled in amounts proportional to milk yield at each milking to make up a single day production sample. A portion of the milk sample was preserved with bronopol-B2 preservative for milk composition analysis, and the other part was frozen at -20 °C and then freeze-dried and vacuum sealed at the Department of Animal Science at the Stellenbosch University for fatty acid analysis by Scaggs Nutrition Laboratories in Utah State University, USA. Samples were analyzed for fat, protein, lactose and milk urea nitrogen by the TAURUS milk analysis laboratory, Irene. Final milk composition was expressed based on weighted a.m. and p.m. milk yields.

Feed samples were collected regularly during the trial to analyze for DM, CP, CF, NDF, ADF, Cu, and ash.

All analytical methods are described in section 3.4 under methodology.

3.3.2   Housing of cows

The cows were housed in a semi open free stall barn in treatment groups. The cows were kept in the same group for the duration of the trial and remained in the same area in the barn to minimize stress.

3.3.3   Feeding program

During the trial period the cows were fed a restricted diet. The diet consisted of a TMR, which was calculated to supply energy and protein for maintenance and to sustain milk production. The TMR was divided into two daily portions, which were presented after milking. A total of 90 kg was allocated per group per day. Consumption of fed rations was recorded on three consecutive days for each period to calculate average intake per group.

3.3.4   Statistical analysis

The data from this study were subjected to a general linear model (GLM) analysis of variance (ANOVA) using STATISTICA 7. Main effects were treatment, cow (group) and period. Means were separated with a Bonferoni test. Significans was declared at $P \leq 0.05$.
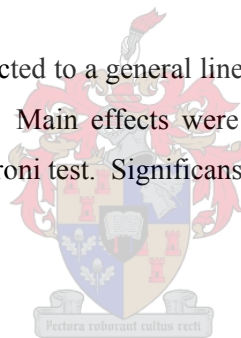
3.4 Analytical Methodologies

3.4.1   Chemical analyses

The analysis for DM, OM, CP and CF contents were according to the guidelines of the AOAC (2000). Neutral detergent fiber and ADF were done according to the method of Van Soest (1967) but using the Ankom[200/220] Fiber Analyzer.

3.4.2   Pasture and feed composition analysis

To reduce the particle size of the samples, the feed and pasture samples were ground to pass through a 0.75 mm screen and stored in air tight sample containers.

3.4.3   Moisture

AOAC Official Method 934.041: Determination of moisture contends of feed.

Dry the clean and labelled porcelain crucible for two hours in oven at 100 °C. Cool in desiccator for 30 min. and record weight. Place crucible onto scale and tare. Weigh 2 g of ground test sample into crucible. Place in oven at 100 °C for 24 hours to dry. Cool in desiccator for 30 min. Record the weight of the dried sample.

% Moisture = **(**(mass of clean dry crucible) + (sample weight) – (weight of dry sample in crucible) / (sample weight)**)** x 100
% DM = 100 - % moisture

3.4.4   Ash

AOAC Official Method 942.05: Ash

Two grams test portions of moisture free feed sample is weighed into a moisture free porcelain crucible and placed into a temperature-controlled furnace at 500 °C for 6 hours. After 6 hours the furnace is switched to 120 °C and the samples are allowed to cool to that temperature. When the desired temperature is reached the samples are transferred directly in a desiccator, cooled and weighed (g). The weight is recorded to the second decimal.

$$\%(\frac{w}{w})\,ash = \frac{Weight\ of\ test\ portion\ [g]\ -\ Weight\ loss\ on\ ashing\ [g]}{Weight\ of\ test\ portion\ [g]} \times 100$$

$$\%\ organic\ material\ (OM) = 100\ \%\ \text{-}\ Ash$$

3.4.5   Crude Protein

AOAC Method 990.03: Crude Protein in Animal Feed

Apparatus:	LECO FP- 528
Accessories:	502-186 Tin Foil Cups
	501-614 Spatula
Sample Weight:	0.20 to 0.35 g
Calibration Standard:	Soya meal
Furnace Temperature:	850 C
Flow Profile:	All High
Protein Factor:	6.25
Analysis Time:	~170 s

Procedure

1. The instrument is operated in accordance with instructions of the manufacturer (i.e., check gas supplies, perform any required maintenance, perform leak checks, etc.).

2. Analyze blanks (gas) until a plateau is reached. Analyze three to five additional blanks and set the blank using these data.

3. Analyze five soya meal standards (using the 502-186 Tin Foil Cups) at 0.10 g and drift correct (if using the PC option).

4. 0.10 g Ground sample is weighed to the nearest 0.01 g into an empty foil sample boat. All weights are recorded. The sample boat is twisted shut to avoid trapping air and placed into the sample tray. The sample is combusted and the % N is obtained. To calculate the % protein of the sample, the % N is multiplied by a protein factor of 6.25 (6.25 is the average % N in plant protein).
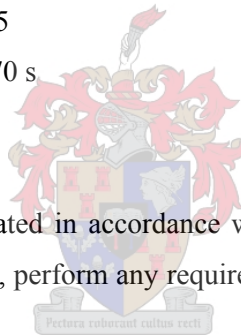
3.4.6   Fat

AOAC Official Method 920.39: Fat (crude) or Ether Extract (EE) in animal feed.

Apparatus: Tecator Soxtec System HT 1043 Extraction Unit

Reagents: Diethyl ether

Method:

1. Place the aluminium fat beakers in oven at 100 °C to dry overnight. Cool in desiccator for 30 min.
2. Weigh and record weight of beakers.
3. Weigh 2 g of ground sample into extraction thimbles. Place a small peace of cotton wool on top of the sample to prevent the sample from boiling out.
4. Fill beakers with approximately 50 ml diethyl ether.
5. Open water tap for condensation before heater is switched on.
6. Switch oil heater and fan on. When heater is ready the thimbles can be placed in the extraction tubes with fat beakers underneath on the element.
7. Lower the extraction tubes and make sure it fits tightly onto the fat beakers. Lower the thimbles into ether and boil for 15 min.
8. Lift the thimbles out of the ether and rinse for 30 min.
9. After rinsing for 30 min. the taps are closed to retain the ether that evaporates.
10. Boil for 15 min.
11. Remove fat beakers from element and place in oven for 2 hours to make sure that all the ether evaporated from the sample.
12. Place fat beakers in desiccator to cool for 30 min.
13. Weigh beakers and record weight.

$$\% \text{ Fat} = \frac{\text{mass of fat beaker after extraction} - \text{mass of moist free beaker}}{\text{sample mass}} \times 100$$

3.4.7   Fiber analysis.

NDF and ADF were determined with the ANKOM[200|220] fiber analyzer ANKOM Technologies, Fairport, NY.

Neutral Detergent Fiber in Feeds

This method determines Neutral Detergent Fiber (NDF) using the filter bag technique, which is the residue remaining after digesting in a detergent solution. The fiber residues are predominantly hemicelluloses, cellulose and lignin.

Apparatus

1. Analytical Balance—capable of weighing 0.1 mg.

2. Oven—capable of maintaining a temperature of correction. 102 ± 2 °C.

3. Digestion instrument—capable of performing the digestion at 100 ± 0.5 °C and maintaining a pressure of 68.9- 172.4 kpa (10-25 psi). The instrument must also be capable of creating a similar flow around each sample to ensure uniformity of extraction (ANKOM[200]; 65 Note—Use sufficient heat to completely seal rpm agitation, ANKOM Technology).

4. Filter bags—constructed from chemically inert and heat resistant filter media, capable of being heat sealed and able to retain 25 micron particles while permitting rapid solution penetration (F57 ANKOM Technology)

5. Heat sealer—sufficient for sealing the filter bags to ensure complete closure (1915; ANKOM Technology).

6. Desiccator pouch—collapsible sellable pouch with desiccant inside that enables the removal of air from around the filter bags (MoistureStop Weigh Pouch, ANKOM Technology).

7. Marking pen—solvent and acid resistant (F08; ANKOM Technology).
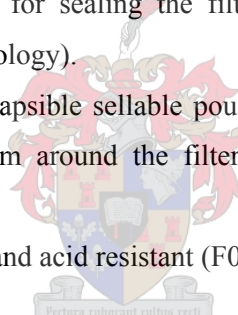
Reagents:

1. Neutral Detergent Solution (ND)—Add 30.00 g sodium lauryl sulfate, USP; 18.61 g Ethylenediaminetetraacetic disodium salt, dihydrate; 6.81 g sodium tetraborate decahydrate; 4.56 g sodium phosphate dibasic, anhydrous; and 10.00 ml triethylene glycol, in 1 l distilled water (ANKOM Technology, premix chemical solution- FND20 or FND20C). Agitate and heat to facilitate solubility. Check pH range to 6.9-7.1.

2. Alpha-amylase - Heat-stable bacterial alpha amylase: activity = 17;40 Liquefon Units/ml (FAA, ANKOM Technology).

3. Sodium sulfite - Na_2SO_3; anhydrous (FSS, 6.ANKOM Technology).

Sample preparation

Grind samples in a centrifugal mill with a 2 mm screen or cutter type (Wiley) mill with an 1 mm screen. Samples ground finer may have particle loss from the filter bags and result in low values.

Procedure:

1. Use a solvent resistant marker to label the filter bag (W1) and zero balance. Note - Do not pre-dry filter bags; any moisture will be accounted for by the blank bag correction.

2. Weigh 0.45-0.55 g of prepared sample (W2) directly in filter bag. Avoid placing the sample on the upper 4 mm of the bag.

3. Using a heat sealer, completely seal the upper edge of the filter bag within 4 mm of the top. Note – Use sufficient heat to completely seal the filter bag and allow enough cool time (2 s) before removing the bag from the heat sealer.

4. Weigh one blank bag and include in run to determine blank bag correction (C1).

5. Pre-extract only samples containing soybean products or $> 5\%$ fat: Extract samples by placing 24 bags with samples into a container with a top. Pour enough acetone into container to cover bag and secure top. Shake the container 10 times and allow bags to soak for 10 min. Repeat with fresh acetone. Pour out acetone and place bags on a wire screen to air-dry. Exception - roasted soybean: Due to the processing of roasted soy, a modification to the extraction is required. Place roasted soy samples into a container with a top. Pour enough acetone into container to cover bags and secure top. Shake the container 10 times and pour off acetone. Add fresh acetone and allow samples to soak for twelve hours. After soak time, pour out acetone and place bags on a wire screen to air-dry.

6. Place a maximum of 24 bags into the Bag Suspender. All nine trays should be used regardless of the number of bags being processed. Place three bags per tray and then stack trays on center post with each level rotated 120 °. Insert the Bag Suspender with bags into the fiber analyzer vessel and place the Bag Suspender weight on top to keep it submerged. Note - Prior to inserting the Bag Suspender, if the vessel temperature is warm from a previous run, add cold water and exhaust.

7. When processing 24 sample bags, add 1900 - 2000 ml of ambient temperature ND solution to the fiber analyzer vessel. If processing less than 20 bags, add 100 ml/bag of ND solution (use minimum of 1500 mL to ensure Bag Suspender is covered). Add 20 g (0.5 g/50 ml of ND solution) of sodium sulfate to the solution in the vessel and 4.0 ml of heat stable alpha-amylase.

8. Turn Agitate and Heat ON and confirm agitation. Set timer for 60 min and close lid.

9. At end of extraction, turn Heat and Agitate off. Open the drain valve (slowly at first) and exhaust hot solution before opening lid. Note—The solution in the

vessel is under pressure. The exhaust valve needs to be opened to release the pressure and solution prior to opening the lid.

10. After the solution has been exhausted, close the exhaust valve and open the lid. Add 1900-2000 ml of (70-90 °C) rinse water. Turn Agitate on and rinse for 5 min. The lid may be sealed with the heat on or left open with the heat off. Repeat 5 min. hot water rinses a total of three times or until water is neutral pH.

11. When the rinsing process is complete remove the samples. Gently press out excess water from bags. Place bags in a 250 ml beaker and add enough acetone to cover bags and soak for 3-5 min.

12. Remove bags from acetone and place on a wire screen to air-dry. Completely dry in oven at $102 \pm 2\,°C$ (most ovens will complete drying within 2-4 hrs). Note—Do not place bags in the oven until acetone has completely evaporated.

13. Remove bags from oven, place directly into a collapsible desiccant pouch and flatten to remove air. Cool to ambient temperature and weigh bags. Note - Do not use conventional desiccator container.

Calculations:

$$\% \text{ NDF (as received basis)} = \frac{W_3 - (W_1 \times C_1)}{W_2} \times 100$$

Where: W_1 = Bag tare weight

W_2 = Sample weight

W_3 = Dried weight of bag with fiber after extraction process

C_1 = Blank bag correction (final oven-dried weight divided by original blank bag weight.)

Notes:

CAUTION

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. Always add acid to water and not the reverse.

CTAB will irritate the mucous membranes. A dust mask and gloves should be worn when handling this chemical.

Acetone is extremely flammable. Avoid static electricity and use a fume hood when handling.

Powdered chemicals will irritate the mucous membranes. A dust mask and gloves should be worn when handling this chemical.

Acid Detergent Fiber

This method determines Acid Detergent Fiber, which is the residue remaining after digesting with H_2SO_4 and CTAB. The fiber residues are predominantly cellulose and lignin.

Method for determining acid detergent fiber (ADF) ANKOM Technology Method 10-21-05 for Acid Detergent Fiber in Feeds with the Filter Bag Technique.

Apparatus:

1. Analytical Balance - capable of weighing 0.1 mg.
2. Oven - capable of maintaining a temperature of correction. 102 ± 2 °C.
3. Digestion instrument - capable of performing the digestion at 100 ± 0.5 °C and maintaining a pressure of 10-25 psi. The instrument must also be capable of creating a similar flow around each sample to ensure uniformity of extraction (ANKOM200; 65 Note—Use sufficient heat to completely seal rpm agitation, ANKOM Technology).
4. Filter bags - constructed from chemically inert and heat resistant filter media, capable of being heat sealed closed and able to retain 25 micron particles while permitting rapid solution penetration (F57 ANKOM Technology).
5. Heat sealer - sufficient for sealing the filter bags closed to ensure complete closure (1915; ANKOM Technology).
6. Desiccator pouch - collapsible sellable pouch with desiccant inside that enables the removal of air from around the filter bags (MoistureStop Weigh Pouch, ANKOM Technology).
7. Marking pen - solvent and acid resistant (F08; ANKOM Technology).

Reagents:

Acid Detergent Solution (AD)—add 20 g cetyl trimethylammonium bromide (CTAB) to 1 l 1.00N H2SO4 previously standardized (premixed chemical solution available from ANKOM). Agitate and heat to aid solution.

Preparation of sample:

Grind samples in a centrifugal mill with a 2 mm screen or cutter type (Wiley) mill with a 1 mm screen. Samples ground finer may have particle loss from the filter bags and result in low values.

Procedure:

1. Use a solvent resistant marker to label the filter bag (W1) and zero balance. Note - Do not pre-dry filter bags; any moisture will be accounted for by the blank bag correction.

2. Weigh 0.45-0.55 g of prepared sample (W2) directly in filter bag. Avoid placing the sample on the upper 4 mm of the bag.

3. Using a heat sealer, completely seal the upper edge of the filter bag within 4 mm of the top. Note - Use sufficient heat to completely seal the filter bag and allow enough cool time (2 s) before removing the bag from the heat sealer.

4. Weigh one blank bag and include in run to determine blank bag correction (C1).

5. Pre-extract only samples containing soybean products or $> 5\%$ fat: Extract samples by placing 24 bags with samples into a container with a top. Pour enough acetone into container to cover bag and secure top. Shake the container 10 times and allow bags to soak for 10 min. Repeat with fresh acetone. Pour out acetone and place bags on a wire screen to air-dry. Exception - Roasted soybean: Due to the processing of roasted soy a modification to the extraction is required. Place roasted soy samples into a container with a top. Pour enough acetone into container to cover bags and secure top. Shake the container 10 times and pour off acetone. Add fresh acetone and allow samples to soak for twelve hours. After soak time, pour out acetone and place bags on a wire screen to air-dry.

6. Place a maximum of 24 bags into the Bag Suspender. All nine trays should be used regardless of the number of bags being processed. Place three bags per tray and then stack trays on center post with each level rotated 120 °. Insert the Bag Suspender with bags into the fiber analyzer vessel and place the Bag Suspender weight on top to keep it submerged. Note - Prior to inserting the Bag Suspender, if the vessel temperature is warm from a previous run, add cold water and exhaust.

7. When processing 24 sample bags, add 1900 - 2000 ml of ambient temperature AD solution to the fiber analyzer vessel. If processing less than 20 bags, add 100 ml/bag of AD solution (use minimum of 1500 ml to ensure Bag Suspender is covered).

8. Turn Agitate and Heat ON and confirm agitation. Set timer for 60 min and close lid.

9. At end of extraction, turn Heat and Agitate off. Open the drain valve (slowly at first) and exhaust hot solution before opening lid. Note - The solution in the

vessel is under pressure. The exhaust valve needs to be opened to release the pressure and solution prior to opening the lid.

10. After the solution has been exhausted, close the exhaust valve and open the lid. Add 1900-2000 ml of (70-90 °C) rinse water. Turn Agitate on and rinse for 5 min. The lid may be sealed with the Heat on or left open with the Heat off. Repeat 5 min. hot water rinses a total of three times or until water is neutral pH.

11. When the rinsing process is complete remove the samples. Gently press out excess water from bags. Place bags in a 250 ml beaker and add enough acetone to cover bags and soak for 3-5 min.

12. Remove bags from acetone and place on a wire screen to air-dry. Completely dry in oven at 102 ± 2 °C (most ovens will complete drying within 2-4 hrs). Note - Do not place bags in the oven until acetone has completely evaporated.

13. Remove bags from oven, place directly into a collapsible desiccant pouch and flatten to remove air. Cool to ambient temperature and weigh bags. Note - Do not use conventional desiccator container.

Calculations:

$$\% \text{ ADF (as received basis)} = \frac{W_3 - (W_1 \times C_1)}{W_2} \times 100$$

Where: W_1 = Bag tare weight
W_2 = Sample weight
W_3 = Dried weight of bag with fiber after extraction process
C_1 = Blank bag correction (final oven-dried weight divided by original blank bag weight.)

Notes:

CAUTION

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. Always add acid to water and not the reverse.

CTAB will irritate the mucous membranes. A dust mask and gloves should be worn when handling this chemical.

Acetone is extremely flammable. Avoid static electricity and use a fume hood when handling.

3.4.8   Milk sample analyses

The samples for milk composition were analyzed with the aid of a Milk- O Scan 133B infrared analyzer at Taurus, Irene, South Africa. The samples were preserved using bronopol-B2 and analyzed for milk fat, protein, lactose and MUN.

Milk samples collected for fatty acid analysis were kept frozen at -20 °C, then freeze-dried, vacuum sealed, and stored at -20 °C. Prior to fatty acid analysis, 3-4 g of the freeze dried milk was mixed with 25 ml of distilled and de-ionized water.

3.4.8.1   Fat extraction

Fat extraction was done according to the method of Hara & Raydin (1978).

Reagents:

1.   Triton   t-octylphenolxypoly-ethoxyethanol

9002-93-1; Sigma Chemical co. St. Louise MO

500ml bottle

2.   Sodium Hexametaphosphate MW 612

203-574-0075; 800-call-1 PB, Pfalz and Baver, Inc.

1 kg bottle

Equipment:

1.   Screw – cap 40 ml (3 x 10) plastic centrifuge tubes
2.   Centrifuge
3.   Boiling water bath (100 °C)
4.   25 ml screw-cap (Teflon lined) test tubes (2 x 150)
5.   5 ml screw-cap (Teflon lined) test tubes
6.   Glass Pasteur pipettes
7.   Vortex

Detergent solution:

1.   Weigh out 30 g Triton and 70 g Sodium Hexametaphosphate.

2. Add 800 ml dH$_2$0 to 1 l beaker and place on stir plate with heater.

3. First add Sodium Hexametaphosphate slowly to the water.

4. Then slowly add Triton.

5. Mix well until dissolved and clear.

6. Add water to make up 1 l.

Procedure:

1. Turn on the boiling water bath (100 °C).

2. Transfer 20 ml of milk sample to labeled centrifuge tube.

3. Centrifuge milk at 2500 rpm (1204 x *g*) for 20 min at 4 °C.

4. Carefully pour the milk/watery part from the tube into the sink leaving as much cream as possible in the tube.

5. Add 20 ml of detergent solution to cream and vortex until the cream is dissolved.

6. Place the tubes in the boiling water bath (100 °C) for 20 min until oil drops appear.

7. Remove oil and white flocculent material with a Pasteur pipette and place in labelled 25 ml screw-cap test tube.

8. Add 10 ml of detergent solution to the oil and white flocculent and vortex until the oil is dissolved.

9. Place back in boiling water bath for 20 min until a yellow/clear layer appears.

10. Remove about 0.1 g of the top oil layer using a Pasteur pipette and transfer to labelled 5.0 ml screw-cap test tubes (the tubes don't need to be weighed).

11. If you don't begin methylation immediately, use parafilm to cap under argon (or nitrogen) and store at - 20 °C.

12. Discard waste.

3.4.8.2   Esterification of fatty acids

For the esterification of methyl esters of milk fat, two well-documented methods are available; the acid-catalysed or the base-catalysed method. It is important to note that the acid-catalysed method, both boron trifluoride/methanol and hydrogen chloride/methanol, is **not** recommended as it causes a considerable amount of isomerization of *cis/trans* bonds to the *trans/trans* configuration (Christy, 1997; Mossoba *et al.,* 1999). The base-catalysed esterification method is therefore essential for the preparation of methyl esters for CLA analysis. This method appears to have no drawbacks, but it is important to note that the reagents used in this method are highly toxic.

Milk FA were transesterified with sodium methoxide solution, prepared according to the method of Christie (1982) with some modifications as described by Chouinard *et al.* (1999). We used this method, as it is the exact method used by the Scaggs Nutrition Laboratory where some of our samples were sent for analysis.

Hexane is used to solubilize polar lipids, methyl acetate is added to minimize competing irreversible hydrolysis reactions, and the mixture is neutralized with oxalic acid when the reaction is complete. The mixture is acidified to prevent hydrolysis during extended periods of storage. Sodium methoxide is used to get a maximum rate of transesterification with minimum hydrolysis. Hydrolysis occurs when trace amounts of water is absorbed from the atmosphere.

Reagents:
1. Hexane
2. Calcium Chloride, anhydrous, pellets

             $CaCl_2$ 110.99 (8-14 mesh)
3. Sodium Methoxide Sodium Methylate,

             124-41-4; Fluka # 71748

             CH_3NaO Mr 54.02

             Keep away from water - highly reactive

             Highly flammable and corrosive

             Causes burns

             Store under nitrogen

             Use ventilation hood

             Wear lab coat and gloves
4. Methanol
5. Methyl Acetate   Highly flammable

             Wear cloves

             Store under nitrogen
6. Diethyl Ether   Toxic

             Extremely flammable

             Store in a dark place

             Use under ventilated hood
7. Oxalic Acid   487 10 20; MERCK

             $(COOH)_2 2H_2O$: 126.07

Equipment:

1. 5ml screw-cap (Teflon lined) test tubes
2. Glass Pasteur pipettes
3. Vortex
4. Centrifuge
5. Boiling water bath (100 °C)
6. Pipette and pipette tips to measure 1.75 ml, 40 μl and 60 μl.

Solutions:

Sodium Methoxide solution-to be made fresh at step 6 of procedure

In a 12 x 75 test tube mix:

1. 1.75 ml Methanol
2. 0.40 ml Sodium Methoxide

Oxalic acid solution- only good for two weeks

1. Weigh 1 g Oxalic Acid into a ceramic crucible and place in oven at 120 °C for 30 min, allow cooling in desiccator.
2. Measure 30 ml Diethyl Ether into labelled amber bottle.
3. Add Oxalic Acid and gently shake until the oxalic acid is dissolved.
4. Store in a dark place.
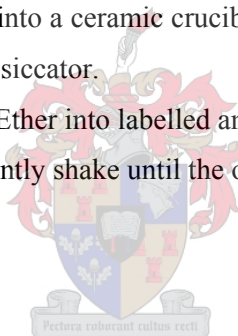
Procedure

IMPORTANT: Many of the reagents are dangerous. Use these under a ventilated hood where specified. Wear gloves and a lab coat.

1. Extract fat as stated above.
2. Melt fat again in water bath if needed.
3. Zero out the weight of small screw cap labelled test tube and add 40 mg of the extracted fat using a Pasteur pipette (about 3 drops).
4. Add 2 ml Hexane and 40 μl methyl acetate (melt fat again if needed).
5. Vortex each sample for 30 s.
6. Make the sodium methoxide solution as described above. Use the ventilation hood. Cover with parafilm between each use.
7. Add 40 μl sodium methoxide solution. Do this under the ventilation hood. Do steps 7 through 9 with only 2 samples at a time.
8. Set timer for 10 min.

9. Vortex each sample for 2 min (2 at a time) immediately after adding sodium methoxide solution. The solution becomes cloudy as sodium-glycerol derivatives are precipitatd.

10. After 10 min, add 60 μl of the oxalic acid solution. This terminates the reaction. Do this under the ventilation hood.

11. Vortex each sample for 30 s.

12. Add a small amount of calcium chloride to each tube (about 10 pellets)

13. Cap and let stand for 1 hour at room temperature.

14. Centrifuge at 2600 rpm (1400 x g) for 5 min at 5 °C leaving a clear layer of hexane.

15. Transfer the clear liquid to a labelled GC vial for analysis. Discard all solutions in an appropriate hazardous waste container.

3.4.8.3 Fatty acid analysis

According to Christie (2002) and Bauman *et al.* (2001), GC analysis of CLA isomers is the method of choice. This method of separation on a 100 m polar column allows reasonable separation of the different $C_{18:2}$ isomers (Kramer *et al.,* 2004). The type of column used is also of importance, as the long flexible fused silica columns coated with highly polar polysiloxane stationary phase, such as the CP- Sil 88; SP 23810; SP 2560; ABD BPX-70, gives much better separation than the carbowax type. With columns that are generally 100 m long, 4 CLA peaks, i.e. in the order 9-*cis*,11-*trans* $C_{18:2}$; 8-*trans,*10-*cis* $C_{18:2}$; 11-*cis*,13-*trans* $C_{18:2}$; 10-*trans*,12-*cis* $C_{18:2}$ are identifiable These are then followed by the *cis / cis* configuration, and then the *trans / trans* groups. Fatty acid peaks are identified by a comparison of the retention times with methylated fatty acid (FAME) standards. The standards used in this investigation are all pure standards of FAME and CLA. The FAME standard used was Supelco 37 from SIGMA and additional CLA standards also obtained from SIGMA.

A more accurate determination of the minor CLA isomers are possible with the use of the tandem-column Ag^{+}-HPLC technique as described by Rickert *et al.* (1999) or single column Ag^{+}-HPLC technique, as described by Nikolova-Damyanova *et al.* (2000) and (Kramer *et al.,* 2004; Mossoba *et al.,* 1999).

The fatty acid % of each fatty acid was calculated by dividing the area under the fatty acid peak by the sun of the areas under the total reported FA. Fatty acids were then converted and expressed as mg/g of FA as is typical for scientific literature.

Gas chromatograph

Column specifications:

Column:	BPX 70
Length:	120 m
ID:	0.25 mm
Film:	0.25 μm

GC Conditions

Initial temp:	130 °C
Ramp 1: 2.0°C/ min	Final temp1: 140 °C
Ramp 2: 1.0°C/ min	Final temp 2: 220 °C
Final time	10 min
Run time:	95 min
Carrier:	Hydrogen
Detector:	FID, 260 °C
Injection: Split	80:1

Injector temperature / Inlet temperature 220 °C

Gas flow rates: hydrogen = 25 ml/min; and hydrogen carrier gas = 2-4 ml/min.

3.5 References

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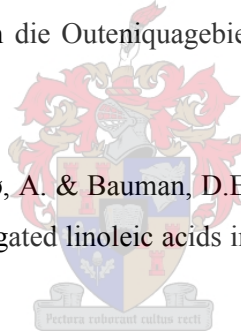
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CHAPTER 4

The Conjugated Linoleic Acid content of milk in South Africa: results from a survey in the Western Cape Province

Abstract

CLA refers to a group of positional and geometric isomers of the omega-6 essential fatty acid, octadecadienoic acid. CLA, and more specifically *cis*-9, *trans*-11C $_{18:2}$, has received considerable attention due to reports of its possible health effects on consumers, whether they are humans or animals. This survey reports on the CLA content of milk from Holstein and Jersey cows on two production systems in the Western Cape, South Africa. Samples were collected from bulk tanks and co-operative silos representing Jersey and Holstein milk from both pasture and TMR-based systems. Samples were analysed for milk and fatty acid composition. Protein and fat content varied between breeds, but not between production systems whilst lactose differed between production systems but not breeds. The CLA levels in the milk from both cow breeds on both production systems were on the low side, but within the range of reported values. CLA levels in milk from the two different production systems differed significantly. Mean CLA level in milk fat of cows from pasture and TMR-based systems were 10.5 and 5.45 mg/g of FA, respectively. Breeds did not differ in the CLA content of milk fat. This is a first report of the CLA levels in milk fat produced in South Africa.

4.1 Introduction

Conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of octadecadienoic acid $C_{18:2}$ with conjugated double bonds (Stockdale *et al*., 2003). Each of these double bonds can either be in the *cis* or *trans* configuration. The biochemical nomenclature for CLA, octadecadienoic acid *cis*-9, *trans*-11 $C_{18:2}$ designates this fatty acid (FA) as an 18 carbon ("octa-deca") FA containing 2 conjugated double bonds ("dien-noic") and specify that the double bonds are found at the 9th and 11th carbon atoms. It also identifies the double bonds being in a *cis* or *trans* configuration.

According to Dhiman *et al.* (2005), a total of 19 natural CLA isomers of $C_{18:2}$ (Table 4.1) have been found in milk, dairy products, beef, human milk, as well as in human adipose tissue.

Table 4.1 Isomers of conjugated linoleic acid $C_{18:2}$

trans-12; *trans*-14$C_{18:2}$		*cis*-12; *trans*-14$C_{18:2}$	
trans-11; *trans*-13$C_{18:2}$	*trans*-11;*cis*-13$C_{18:2}$	*cis*-11; *trans*-13$C_{18:2}$	*cis*-11; *cis*-13$C_{18:2}$
trans-10; *trans*-12$C_{18:2}$	*trans*-10; *cis*-12$C_{18:2}$	*cis*-10; *trans*-12$C_{18:2}$	
trans-9; *trans*-11$C_{18:2}$		*cis*-9; *trans*-11$C_{18:2}$	*cis*-9; *cis*-11$C_{18:2}$
trans-8; *trans*-10$C_{18:2}$		*cis*-8; *trans*-10$C_{18:2}$	
trans-7; *trans*-9$C_{18:2}$	*trans*-7; *cis*-9$C_{18:2}$	*cis*-7; *trans*-9$C_{18:2}$	
trans-6; *trans*-8$C_{18:2}$		*cis*-6; *trans*-8$C_{18:2}$	

Biomedical studies with animal models, and some human studies, have shown that CLA possesses beneficial health effects to both animal and human life (McGuire & McGuire, 2000; Devery *et al.*, 2001; Kelly, 2001; Pariza *et al.*, 2001; Parodi, 2003; Weiss *et al.*, 2004). Some of these include anticarcinogenic, antiatherogenic, antiobesity, immune system enhancement and antidiabetic effects. CLA is preferentially found in food products derived from ruminant animals (Kramer *et al.*, 1998). Thus, dairy products are one of the major dietary sources of CLA. Different CLA isomers may be responsible for different health effects and it has been established that *cis*-9, *trans*-11 $C_{18:2}$ (termed rumenic acid (RA)); Kramer *et al.*, 1998), is anticarcinogenic when included in the diet as a natural food component (Ip *et al.*, 1999; Parodi, 2003). Rumenic acid is the major CLA isomer in dairy products and may contribute up to 94 % of the total CLA isomers present in bovine milk (Chin *et al.*, 1992; Parodi, 1999; Kelly, 2001; Dhiman *et al.*, 2005). It is therefore understandable that the formation of CLA, the modes of action, and possible enhancement of CLA (specifically RA) in ruminant products, are receiving considerable attention.

It has been confirmed that RA can be formed both ruminally by microbial activity during biohydrogenation of linoleic acid (LA), and endogenously in the mammary gland from vaccenic acid (VA), which is an intermediate product of ruminal biohydrogenation (Parodi, 2003; Khanal & Dhiman 2004). Dietary lipids are rapidly hydrolyzed in the rumen, resulting in free unsaturated FA that is subjected to biohydrogenation by rumen micro-organisms. As a consequence, ruminants absorb mainly saturated FA, and the food products derived from ruminants tend to have a more saturated FA composition, regardless of the FA composition of the diets.

In ruminants, RA is synthesized as a relatively stable intermediate in biohydrogenation of dietary LA by linoleic acid isomerase, produced by the rumen bacteria *Butyrivibrio fibrisolvens*. The process occurs in two steps of which the first is formation of a conjugated diene. In the second step the conjugated diene is further hydrolyzed to the *cis*-9, *trans*-11 $C_{18:2}$ (RA). Further biohydrogenation would result in *trans*-11 $C_{18:1}$ (VA) and then stearic acid $C_{18:0}$. However, when biohydrogenation of $C_{18:2}$ and $C_{18:3}$ is not complete, RA and VA escapes from the rumen and can be absorbed from the gastro intestinal tract, thereby providing the mammary gland with a source of RA and VA (Ellen & Elgersma, 2004; Destaillats *et al.,* 2005).

Rumenic acid can also be endogenously synthesized from VA by the Δ^9 desaturase enzyme (Griinari *et al*., 1998; Corl *et al*., 2001; Peterson *et al*., 2002; Fievez *et al*., 2003). Vaccenic acid escapes the rumen either as intermediates from the biohydrogenation of LA or as a product from the biohydrogenation of linolenic acid (LNA). Synthesis of CLA through the endogenous pathway has been estimated to account for 91 % of total CLA in milk fat (Griinari *et al*., 2000; Corl *et al*., 2001; Kay *et al*., 2004).

The CLA content of milk may vary substantially between cows on the same diet, as shown by Peterson *et al*. (2002) in a study with Holstein cows. It was evident from their study that even though diet plays an important role in milk fat content and milk fat composition, individual animal differences are also important. The variation between cows is due to differences in the rumen microbial population and, therefore, difference in rumen biohydrogenation, as well as a variation in the rate of Δ^9 desaturase activity of the mammary gland, which will greatly affect endogenous synthesis.

The objective of this study was to determine the CLA concentration present in bovine milk from breeds and production systems that are commonly used in the Western Cape Province of South Africa. This project was conducted to provide a reference point for CLA concentration in milk bulk tanks from Holstein and Jersey herds that are either grazing pastures or that are kept on TMR feeding systems.

4.2 Materials and methods

Milk samples were collected from bulk tanks of 24 dairy herds and from 3 co-operative silos to determine the CLA levels of milk in the Western Cape. The 24 bulk tank samples were collected from six Holstein and six Jersey herds on zero-grazing systems as well as on TMR-based systems. The TMR's of cows on the zero-grazing systems consisted of typical high concentrate diets (at least 60% concentrate), while pastures consisted predominantly of lush ryegrass with varying levels of kikuyu or clover. Silo milk samples were from a mixed herd milk silo and a Jersey herd milk silo containing milk from TMR-based cows in the Stellenbosch area and from a mixed herd silo containing milk from pasture-based cows in the Mossel Bay area.

Each milk sample (250 ml) was divided into 2 sub samples, one for the determination of protein, fat and lactose, and the other for the determination of fatty acid composition. The samples for milk composition were analyzed with the aid of a Milk-O-Scan 133B at the ANPI Dairy Laboratory at Elsenburg, Stellenbosch. Milk samples for fatty acid analysis were freeze-dried and vacuum-sealed at the Department of Animal Sciences, Stellenbosch University, and sent to the Skaggs Nutrition Laboratory at the Utah State University, USA, for quantifying FA using gas chromatography.

All analytical methods are described in Chapter 3, section 3.4 under methodology.

4.3 Statistical analysis

Data were subjected to a one way ANOVA with the aid of the GLM procedure of SAS 8.02 (2000). Least square means were determined and a Bonferoni test was used to separate means. Significance was declared at $P \leq 0.05$.

4.4 Results and discussion

Results of milk composition and CLA content in milk from the survey are in Table 4.2

Jersey milk had a higher protein and fat content than Holstein milk, but the feeding system had no effect on protein or fat. Similarly, the lactose content did not differ between breeds,

but was higher in milk from the pasture-based cows. In this study, breed had no effect on the total CLA content. Although the CLA content of milk from pasture-based cows was higher than that from TMR-based cows, the levels were within the range reported in the literature for pasture and TMR.

The milk content of total VA from cows on pasture was higher than that of TMR-based cows. It has been reported in the literature that VA levels in milk may be 2-3 fold higher than RA levels. In the current study, VA levels were almost 4 fold higher than RA levels. The RA content of milk from pasture-based cows is usually much higher than that of TMR-based cows (Jahreis *et al.,* 1996). This was also observed in the current study.

Table 4.2 Milk composition of bulk tank and silo samples obtained from Holstein and Jersey cows on TMR-based or pasture-based systems

Item	TMR-based		Pasture-based		P
Bulk tank samples	Holstein	Jersey	Holstein	Jersey	
Protein (%)	3.15[a]	3.69 [b]	3.23 [a]	3.56[b]	<0.001
Fat (%)	3.51 [a]	4.40 [b]	3.62 [a]	4.41[b]	<0.001
Lactose (%)	4.82 [a]	4.91 [a]	4.67 [b]	4.62[b]	<0.001
$C_{18:1}$ *trans*-11 (VA) (mg/g FA)	17.9 [a]	19.6 [a]	33.0[b]	39.8[b]	<0.001
$C_{18:1}$ *trans*-9 (mg/g FA)	6.3	6.8	9.2	8.3	NS
Total $C_{18:1}$ *trans* (mg/g FA)	24.1 [a]	26.4 [a]	42.2[b]	48.1[b]	<0.004
cis-9; *trans*-11$C_{18:2}$ (RA) (mg/g FA)	4.6 [a]	4.3 [a]	10.0[b]	10.7 [b]	<0.001
trans-10; *cis*-12 C $_{18:2}$ (mg/g FA)	0.5	1.5	0.3	0.5	NS
Total CLA[1] (mg/g FA)	5.1 [a]	5.8 [a]	10.3[b]	11.2 [b]	<0.001
Total CLA (mg/kg milk)	179.0 [a]	255.8[2a]	372.8[b]	493.9[b]	<0.001
Silo samples	Mixed herd TMR		Jersey herd TMR	Mixed herd Pasture	
Total CLA (mg/g FA)	4.1		4.7	8.8	

[a,b] Means in the same row with different superscripts differ (P≤ 0.05)

[1] Total of *cis*-9, *trans*-11C $_{18:2}$ and *trans*-10, *cis*-12 C $_{18:2}$

Previous studies demonstrated that the milk concentration of CLA was dependant on the presence of unsaturated FA in the diet (Dhiman *et al*., 1999a,b), especially LA and LNA (Dhiman *et al*., 2000), and that the CLA content of cow's milk can therefore be increased through a diet that contains high levels of these FA, or by using supplements with a high $C_{18:2}$ and $C_{18:3}$ content. Milk fat is the richest natural source of CLA with typical concentrations raging from 2.4-28 mg/g of fat, but this value may vary widely between herds, breeds (Jahreis *et al.,* 1996, Kelly *et al.,* 1998a; 1998b; Lawless *et al*., 1998; Morales *et al.,* 2000a,b) different feeding and management practices (Dhiman *et al*., 1999a,b; 2000; 2001; 2005;

Fritsche *et al.,* 1999; Peterson *et al*., 2002), individual animals (Jiang *et al*., 1996; Kelly *et al*., 1998a,b; Lawless *et al*., 1998; Peterson *et al.,* 2002) and different seasons (Lock *et al*., 2003). It has also been documented that the copper status of the animal may significantly influence the CLA synthesis (Morales *et al.,* 2000a,b)

The *trans*-10; *cis*-12 C $_{18:2}$, is known for milk fat depression (MFD) (Baumgard *et al*., 2002; Parodi, 2003) and even though the levels of this fatty acid was not statistically different between breeds, the Jersey milk still contained more milk fat than the Holstein milk. This difference in milk fat is common for the breeds.

Total CLA values mg/g of FA in the milk from the silo samples were 4.1; 4.7 and 8.8 for the mixed TMR, Jersey TMR and the mixed pasture based systems, respectively. Milk CLA values of the silo samples followed a similar trend as discussed above (higher CLA levels from pasture-based cows), but values were lower than that in milk collected from individual herd bulk tanks.

4.5 Conclusions

The purpose of this study was to quantify the CLA content of milk in South Africa. The levels of CLA in milk from both TMR and pasture-based systems are within the range reported from other countries. It also follows a similar trend as reported with cows on pasture having higher levels of CLA in milk fat than cows on zero-grazing systems.
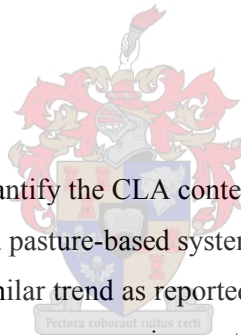
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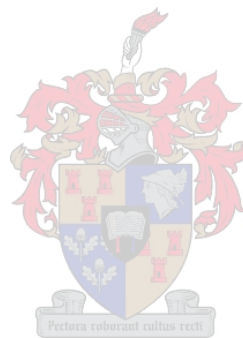
CHAPTER 5

Effects of Conjugated Linoleic Acid (CLA) supplementation to grazing dairy cows on the CLA content of milk

Abstract

Forty multiparous Jersey cows averaging 78 (125-157) DIM were used in a pasture based experiment to determine effects of a CLA supplement on the CLA content of milk. The cows, two homogeneous groups of 20, were kept on pasture and supplemented with 6 kg concentrates/day for a 100 day period. Treatments consisted either of the control concentrate, or with concentrate containing an additional 70 g/day of CLA. Milk samples were obtained every 14 days and analyzed for milk composition and fatty acid composition. Treatment had no effect on milk production, milk yield, % protein, % lactose, and MUN. Percentage milk fat and milk fat yield were decreased by CLA supplementation. The MFD can be contributed to a higher milk content of *trans*-10, *cis*-12 $C_{18:2}$ CLA in the treatment group. Milk content of *cis*-9, *trans*-11 $C_{18:2}$ CLA were 1.2-1.4 fold greater in the treatment group than the control. This experiment indicated that a CLA supplement increases the milk content of *cis*-9, *trans*-11 $C_{18:2}$ CLA, the most biologically active isomer of $C_{18:2}$ CLA, but caused severe MFD.
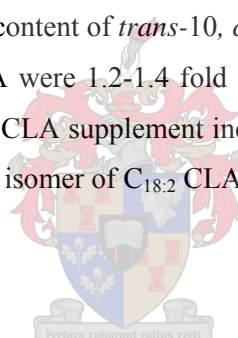
5.1 Introduction

Conjugated linoleic acid refers to a collection of isomers of octadecadienoic acid ($C_{18:2}$) which contains two conjugated double bonds. These double bonds are found in positions; 7 & 9; 8 & 10; 9 & 11; 10 & 12; 11 & 13; 12 & 14 and can be in any of the following geometric configurations; *cis / cis, cis / trans, trans / cis, trans / trans* (Stanton *et al.*, 1997; Rickert *et al.*, 1999; Aydin *et al.*, 2005). Conjugated linoleic acid (CLA) is found predominantly in ruminant derived food products (Chin *et al.*, 1992; Aydin *et al.*, 2005; Dhiman *et al.*, 2005). Milk contains levels of 2.4 – 28.1 mg CLA / g of FA (FA). This is up to 5 times the amount found in ruminant meat (Chin *et al.*, 1992). The most biologically active isomer of CLA is *cis*-9, *trans*-11 $C_{18:2}$, also termed rumenic acid (Kramer *et al.*, 1998; Ellen and Elgersma, 2004; Destaillats *et al.*, 2005), is also the most abundant isomer, and milk fat contains levels of up to 94 % of this isomer (Dugan *et al.*, 2001; Collomb *et al.*, 2004; Dhiman *et al.*, 2005).

CLA has been under intensive investigation in recent years due to reports of possible health benefits to humans, as well as animals (Bauman *et al.*, 2001; Parodi, 2003). These potential benefits include

possible anticarcinogenic effects, antiatherogenic, immunomodulating, growth promoting and lean body mass-enhancing properties, normalized impaired glucose tolerance in non-insulin-dependent diabetes, modulatation of food allergic reactions, the reduction in growth of melanoma, leukaemia, mesothelioma, and glioblastoma together with breast, prostate, colon, and ovarian cancer as well as two human hepatoma cell lines (Cook *et al.*, 1993; Chin *et al.*, 1994; Banni *et al.*, 1996; Parodi, 1997; Parodi, 1999; Ip *et al.*, 1999; Pariza *et al.*, 2001; Weiss *et al.*, 2004a; Weiss *et al.*, 2004b; Selberg *et al.*, 2004; Aydin *et al.*, 2005). Of all the possible health benefits, the anticarcinogenic effect of rumenic acid (RA) has been the most extensively investigated and the National Academy of Science indicated that CLA is the only fatty acid to unequivocally suppress carcinogenesis in experimental animals.

These findings sheds new light on milk as a functional food (i.e., foods with more than just nutritive value) rather than a harmful food. Historically, milk has spent many years under the public eye due to its high content of undesirable FA.

To increase human uptake of CLA, humans need to either consume more foods containing CLA, or increase the content of CLA in the products being consumed. The latter response is more practical as a wide range of milk products is already available to consumers. The CLA content of milk varies greatly among different management practices, pasture vs. TMR, (Jahreis *et al.*, 1996; Dhiman *et al.*, 2005; Current study, Chapter 4), animal breeds (Lawless *et al.*, 1999; Whitlock *et al.*, 2002; Dhiman *et al.*, 2005; Current study, Chapter 4), seasons, as well as among individual animals (Kelley *et al.*, 1998a; Kelley *et al.*,1998b; Peterson *et al.*, 2002). CLA is synthesized (Harfoot & Hazelwood, 1988; Enser *et al.*, 1999; Offer *et al.*, 1999; Donovan *et al.*, 2000; Griinary *et al.*, 2000) in the rumen during biohydrogenation of linoleic acid (LA). On the other hand, vaccenic acid (VA) is synthesized during biohydrogenation of LA and linolenic acid (LNA). Vaccenic acid (VA) acid in turn provides substrate for endogenous synthesis of CLA in the body tissues. This is accomplished through the activity of Δ^9 desaturase enzyme activity. By feeding animals feeds rich in LA (*cis*-9*, cis*-12 $C_{18:2}$), LNA, (*cis*-9*, cis*-11*, cis*-15 $C_{18:3}$) and VA (*trans*-11 $C_{18:1}$), all substrates for CLA synthesis, the CLA content in milk may be greatly increased (Stanton *et al.*, 1997; Dhiman *et al.*, 1999a; Griinari and Bauman, 1999; Bessa *et al.*, 2000).

In pasture based management systems in South Africa, pasture is normally supplemented with concentrate to ensure sufficient supply of energy and protein to support the cow for maximum milk production. This practice is commonly used in South Africa as pasture cannot always supply in the energy- and protein requirements of high yielding dairy cows (Du *et al.*, 1996; Schroeder *et al.*, 2003). Compared to TMR diets, pasture based diets have several differences that relate to milk fat CLA. The RA and CLA concentrations in milk from cows on pasture diets are typically higher than those of

milk from cows on TMR diets (Bauman *et al*., 2001). Green pastures contain substantial amounts of LNA while preserved forages contain more LA. Rumen microbial organisms involved in biohydrogenation may be affected by pasture based diets as the rumen pH is usually lower in pastured cows; also, passage rate is typically higher in cows on pasture based diets (Agenäs *et al*., 2002; Kay *et al*., 2004; Qiu *et al*., 2004a).

Formulating supplemental concentrates with fats high in LA, LNA, and VA (e.g. sunflower oil, soybean oil, cottonseed oil, linseed oil, rapeseed oil, or there respective seeds), will usually increase the CLA content of milk (Offer *et al*., 1999, 2001; Dhiman *et al*., 2000; Whitlock *et al*., 2002). Dietary fat may have a favourable fatty acid composition for milk synthesis, but this is normally not reflected directly in the milk composition due to extensive biohydrogenation in the rumen, as well as the desaturase activity in the tissues. A large amount of dietary polyunsaturated fatty acids (PUFA) are saturated in the rumen and some of the saturated FA is then desaturated to their respective mono-unsaturated FA by desaturase enzyme activity in the tissues. CLA is formed as an intermediate in both these processes. If biohydrogenation in the rumen is inhibited, larger amounts of CLA and VA will escape from the rumen. This leads to even more CLA being synthesised in the tissues from the VA escaping the rumen (Harfoot & Hazelwood, 1988; Enser *et al*., 1999; Griinari *et al*., 2000; Agenäs *et al*., 2002; Kelsey *et al*., 2003).

In the current trial, a CLA supplement was used to determine the effect of CLA supplementation on milk production and milk composition of pastured dairy cows.
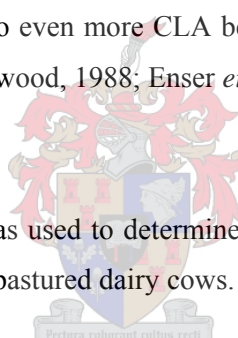
5.1 Materials and methods

The experiment was conducted at the Outiniqua experimental farm in George, South Africa, in the last quarter of 2004.

5.1.1 Animals

Forty multiparous Jersey cows, 25-157 DIM were allocated to one of two groups. Although the cows were allocated to specific groups, they were kept together as one herd to minimize differences in environmental influences. Groups were balanced for days in milk (DIM), milk production (MP) and lactation number to ensure homogeneity. Means of the groups were DIM 78 ± 38.12, lactation number 4.4 ± 2.02 and MP 21.5 ± 2.13 kg/day. The milk production data was based on the average of a three-week production period, two weeks before the commencement of the trial.

5.1.2 Sampling and chemical analyses

The experimental period was 12 weeks; the first two weeks of the experiment served as a pre-trial period that allowed the cows to adapt to the new diets and the next 10 weeks as a trial period. Data collections were made during weeks 4, 6, 8, 10 and 12. Data collections were always on Tuesdays and Wednesdays, at fixed times and by the same person to enhance accuracy and ensure consistency.

The cows were weighed bi-weekly and the body condition was scored on a scale of 0-5. To ensure accurate body mass values, the cows were weighed on two consecutive days just before the afternoon milking.

The herd had 24 hours access to pasture and each cow received 6 kg of a high-energy concentrate per day. The concentrate was balanced for minerals and vitamins, and was formulated to meet the nutrient requirements of lactating cows according to NRC recommendations (NRC, 2001). The Cornell Net Carbohydrate and Protein System (CNCPS) was used to formulate the concentrate. The chemical composition of the concentrate feed is presented in Table 5.1. Fresh water was available at all times during grazing.

Table 5.1 Calculated mean nutrient composition of the concentrate supplemented on pasture.

Mean nutrient composition	
DM %	89
Crude Fat %	4
Crude Prot %	11
NDF %	9
ADF %	4
Ash %	5
Cu mg/kg	30.6
Energy (calculated)	15MJ ME/kg (DM)

The concentrate used in the trial for both control and CLA groups was a commercial concentrate and was supplied by Molatek (P.O. BOX 343, Mokopane, 0600). The concentrate was fed in two equal portions of 3 kg during the morning and afternoon milkings. The CLA supplement was hand mixed into the concentrate after being measured out for each cow.

The two groups grazed as one herd on pasture strips that were allocated daily according to DM yield/ha. The average daily pasture allowance was estimated to be 10 kg DM/cow/day. The DM yield of the pasture was calculated with the aid of height measurements taken with a rising plate

pasture meter, and a regression was calculated using the relationship between compressed sward height and herbage mass. The compressed sward height was determined by taking 100 readings per paddock with a rising plate meter. To calculate the regression, pasture samples was cut weekly, dried and the DM content was then plotted on a graph. A regression line was fitted which allowed the prediction of DM yield with reasonable accuracy. The metal ring had a surface area of 0.0999 m^2 (Ø = 357 mm) and all the herbage inside the ring was cut to a height of 3 cm from the ground. Dried pasture samples from each week of the trial period were analyzed for DM, CP, NDF, ADF, Cu and ash according to AOAC (2002) methods. Results are presented in Table 5.2. The pasture consisted mostly of a grass mixture of kikuyu that was sown over with ryegrass during the winter. At the start of the trial in September the herbage consisted predominantly of ryegrass but as temperatures increased during spring, the kikuyu started to grow actively. When the trial ended in December, the pasture was still predominantly ryegrass but with a considerable amount of kikuyu.

Table 5.2 Change in pasture composition during the trial period.

Week	DM %	Crude Fat % of DM	Crude Protein % of DM	NDF % of DM	ADF % of DM	Ash % of DM	Copper mg/kg of DM
2	11.7	4	23.7	47.2	29	11.9	5.6
3	11.5	4	26.7	42.8	27	13.3	7.2
4	11.2	4	20.2	37.4	25	8.6	6.8
5	16.9	4	23.0	45.9	27	11.9	5.7
7	12.5	4	27.5	52.1	28	12.7	6.1
8	14.0	4	23.7	49.8	26	11.3	6.5
9	18.3	3	22.2	51.0	27	11.3	5.7
10	21.3	3	20.7	52.8	28	9.4	6.5

Milk yield of each cow was recorded electronically twice daily. Every second week (starting in week 4), afternoon and morning milk samples were collected on two consecutive days at a rate of 10 ml/L milk produced. Part of the milk sample was preserved with bronopol-B2 preservative for milk composition analysis, and the other was frozen at -20°C and then freeze-dried and vacuum-sealed at the Department of Animal Sciences, Stellenbosch University, for fatty acid analysis. The preserved samples were analyzed for fat, protein, lactose and milk urea nitrogen by the TAURUS milk analysis laboratory, Irene. Final milk composition was expressed based on weighted a.m. and p.m. milk yields. Milk samples collected during the first month for fatty acid analysis were sent to the Skaggs Nutrition Laboratory at the Utah State University (USA). However, when the rest of the batches (last two periods) were sent, they were returned by USA Immigrations due to restrictions on samples from

animal origin that had been installed in the USA in the interim period. The samples were therefore analyzed in the laboratory of the Department of Animal Sciences at the Stellenbosch University.

Milk fat extraction was done according to the method of Hara & Radin (1978). The milk was reconstituted by adding 25 ml of de-ionized distilled water to 3-4 g of freeze-dried milk. After thorough mixing, the milk was centrifuged at 2500 rpm (1204 x g) for 20 min. at 4 °C. The fat cake was removed and 20 ml of detergent solution (30 g Triton and 70 g sodium hexametaphosphate in 1000 ml de-ionized distilled water) was added. The mixture was vortex until the cream dissolved and placed in a boiling water bath (100 ˚C) for 20 min. The oil and flocculent layer was removed, added to another 10 ml of detergent solution, vortexed, and placed back into the water bath for 20 min. Approximately 0.1 g of the fat layer was removed, capped under nitrogen and stored at -20 ˚C until analysed for FA.

Milk FA were transesterified with sodium methoxide according to the method of Christie (1982), with modifications. A volume of 2 ml hexane was added to 40 mg of the extracted fat, followed by 40 µl of methyl acetate. After vortexing the mixture for 30 seconds, 40 µl of the methylation reagent (1.75 ml methanol: 0.4 ml of 5.4 M sodium methylate) was added. The mixture was immediately vortexed for 2 min. and allowed to react for a total time of 10 min. To stop the reaction, 60 µl of termination reagent (1 g oxalic acid/30 ml of diethyl ether) was added. A small amount of calcium chloride was added to each tube and the samples were incubated at room temperature for one hour before centrifuged at 2600 rpm (1400 x g) for 5 min at 4 ˚C. The hexane supernatant was removed and used directly for gas chromatographic determination.

Fatty acid methyl esters were analyzed by gas chromatography (Hewlett Packard GC system 6890+ with a flame ionizing detector) using a BPX 70 capillary column (120 m x 0.25 mm i.d. with 0.25 µm film thickness). The FAME standard used was Supelco 37 from SIGMA and additional CLA standards also obtained from SIGMA. A programmed run with a total run time of 95 min. was used. Gas chromatograph conditions were as follows: initial temp = 130 °C; 1st ramp = 2.0 °C/min with a final temperature of 140 °C; 2nd ramp = 1.0 °C/min with a final temperature of 220 °C; final time = 10 min. Injector temperature was set at 220 °C. Gas flow rates were: hydrogen = 25 ml/min; and hydrogen carrier gas = 2-4 ml/min. An injector split of 80:1 was used with the flame ionizing detector at 260 °C.

The fatty acid % of each fatty acid was calculated by dividing the area under the fatty acid peak by the sum of the areas under the total reported FA. Fatty acids were then converted and expressed as mg/g of FA as is typical for scientific literature.

To monitor the accuracy of fatty acid determination, 10 samples were prepared by a lab assistant, according to the method described above. The samples were then analysed for fatty acid profiles at the Department of Animal Sciences, as well as the Department of Organic Chemistry, Stellenbosch University. In both departments, all 10 samples were done with a GC under similar conditions as described above. The department of Organic Chemistry did an additional analysis by using mass spectophotometry. The samples were compared and no difference ($P > 0.05$) was found between methods or departments for any of the samples or FA in the separate analyses. We therefore concluded that our method of fatty acid profile determination was correct and repeatable.

To assess desaturase activity, the desaturase index was calculated for CLA. In addition to CLA Δ^9 desaturase index, another three pairs of FA exist in milk fat that represents products and substrates for Δ^9 desaturase (Kelsey *et al*., 2003). The desaturase index is calculated as follows: product of Δ^9 desaturase / (product Δ^9 desaturase + substrate Δ^9 desaturase), and was calculated for the following fatty acid pairs: *cis*-9 $C_{14:1}/C_{14:0}$, *cis*-9 $C_{16:1}/C_{16:0}$, *cis*-9 $C_{18:1}/C_{18:0}$ and *cis*-9, *trans*-11$C_{18:2}$ /*trans* 11 $C_{18:}$.

5.2 Statistical analysis

Cows were stratified according to milk production, days in milk (DIM) and lactation number and then randomly divided into two groups of 20, resulting in two comparative groups. For each data collection period, the data were subjected to a one-way analysis of variance (ANOVA) using STATISTICA 7. Where appropriate, repeated measures ANOVA's were completed. Differences in milk yield were determined based on data gathered during the whole feeding period, while milk composition was compared in samples collected on a two weekly bases. The samples compared for fatty acid profiles were based on data collected monthly.

5.3 Results and discussion

The two groups of dairy cows were homogenous at the start of the trial as indicated in Table 5.3 for days in milk (DIM), lactation number and milk production (MP). The MP data is based on an average MP kg/day for three weeks before the commencement of the trial. There is some contradiction in literature as to whether these parameters should be included in the design of the experiment or not. According to Lock *et al*. (2005), DIM, lactation number and MP has no effect on CLA content of milk, however, Kelsey *et al*. (2003) found that lactation number has an effect on CLA production. In the layout of this trial the groups were homogenous to minimize the affect, if any, of these parameters.

Table 5.3 Least square means (±SEm) for DIM, lactation number and milk production of cows in treatment groups at the start of the trial.

Production characteristic	Control	CLA	Homogeneity of variance (P)
DIM	80.65 ± 8.38	81.64 ± 8.6	0.293
Lactation nr.	4.10 ± 0.45	4.47 ± 0.46	0.798
MP	21.55 ± 0.47	21.31 ± 0.47	0.768
N	20	19	

Treatment means for milk yield and milk composition for the 100 days period are presented in Table 5.4. Treatment had no effect on weekly milk production or total milk yield. The CLA supplement caused MFD with a group mean for milk % fat of 4.09 and 3.45 for the control and CLA group, respectively. Supplementation of CLA had no effect on milk protein, lactose or MUN content. The weekly average values for milk fat, protein, lactose and MUN are presented in figures 5.2-5.5.

Table 5.4 The effect of CLA supplementation to pasture-based Jersey cows on milk production and milk composition.

Item	Treatment (Mean ± SEm)		P
	Control	CLA	
Milk yield (kg)	1640.44 ± 37.33	1657.49 ± 38.30	0.752
Milk fat %	4.09 [a] ± 0.95	3.45 [b] ± 0.09	**0.001**
Prot %	3.18 ± 0.05	3.06 ± 0.04	0.642
Lactose %	4.61 ± 0.03	4.69 ± 0.03	0.062
MUN mg/dL	13.80 ± 0.35	13.18 ± 0.33	0.206
Milk fat yield (kg)	64.47 a ± 1.71	56.87 b ± 1.75	**0.004**
Protein yield (kg)	50.86 ± 1.35	50.98 ± 1.38	0.948
Lactose yield (kg)	73.70 ± 1.95	78.02 ± 2.00	0.131
N	20	19	

[a,b] Means in the same row with different superscripts differ (P≤ 0.05)

There was no week x treatment interaction in MP (Table 5.5). The effect of week (time) on MP can be seen in Figure 5.1. This effect could be expected as milk production followed a normal lactation curve over time. The P-values for weekly and monthly milk production are presented in Table 5.5.

Table 5.5 Level of significance for average milk production.

Average milk production	Week	Month
	P	P
Treatment	0.752	0.715
Time	**0.001**	**0.001**
Treatment x Time	0.245	0.175

There was no treatment x month interaction for MP, but once again, there was an expected decrease in MP over time. The effect of CLA supplementation on milk production over time (kg/day per month) is displayed in Table 5.6.

Table 5.6 The effect of CLA supplementation on milk production over time (kg/day by month) of pasture-based Jersey cows.

	Month 1		Month 2		Month 3	
	Mean ± SEm		Mean ± SEm		Mean ± SEm	
Treatment	Control	CLA	Control	CLA	Control	CLA
Daily milk production by month	21.00 ± 0.47	21.00 ± 0.48	19.00 ± 0.51	19.62 ± 0.52	18.32± 0.40	18.40 ± 0.41
N	20	19	20	19	20	19
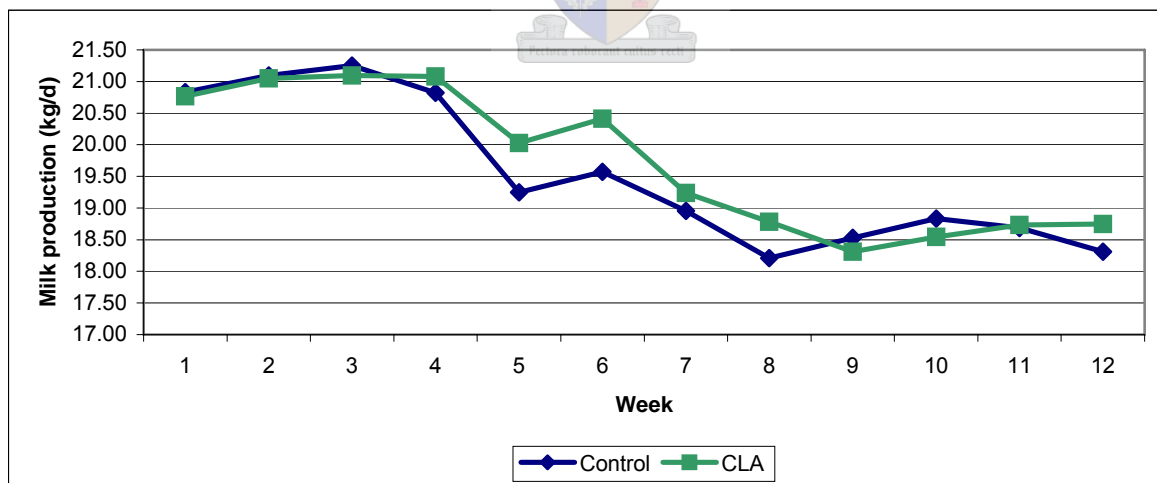
Figure 5.1 The effect of CLA supplementation to pasture-based Jersey cows on weekly mean milk production (kg/day over 12 weeks)

The effect of CLA supplementation on weekly mean milk fat content is presented in Figure 5.2.
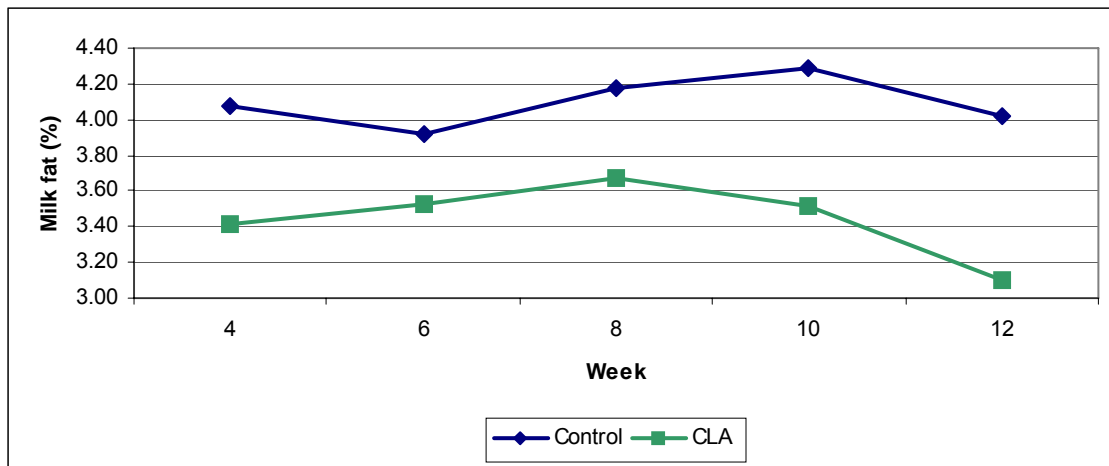
Figure 5.2 The effect of CLA supplementation to pasture-based Jersey cows on weekly mean milk fat percentage (over 12 weeks)

From Figure 5.2 it is evident that the effect of CLA treatment on milk fat content remained fairly constant over time and those fluctuations followed the same trend in both groups. The fat suppressing effect of the CLA treatment is clearly visible and appeared to have increased over time. Milk fat depression is a well-known occurrence where diets are supplemented with fats. With the supplementation of diets with CLA, a definite relationship has been found between MFD and CLA (Griinari & Bauman, 1999; Baumgard *et al.*, 2000, 2001; Bauman *et al.*, 2001).

The effect of treatment on milk protein content over time is presented in Figure 5.3.
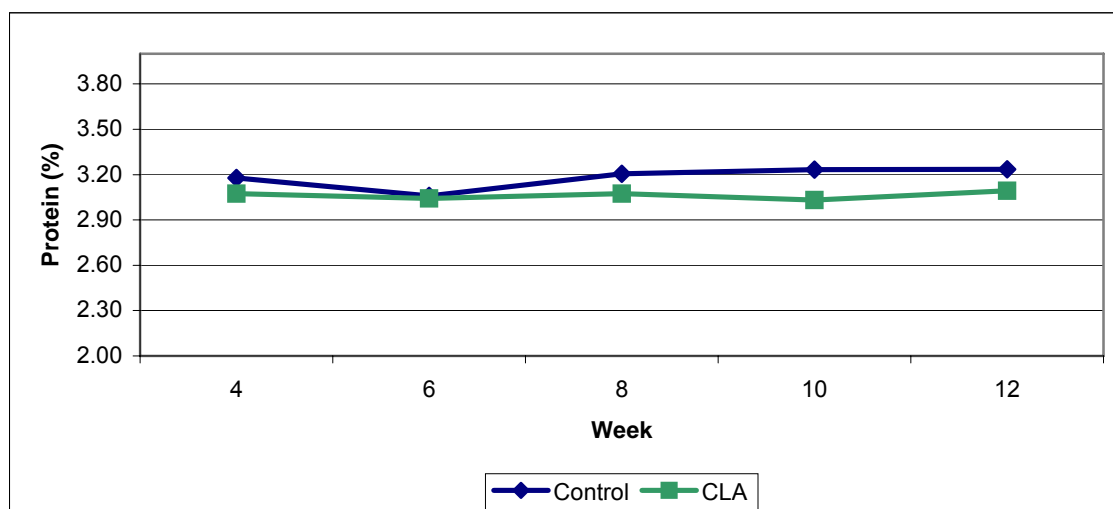
Figure 5.3 The effect of CLA supplementation to pasture-based Jersey cows on weekly mean milk protein percentage (over 12 weeks).

From Figure 5.3 it is clear that the milk protein content did not follow the same trend as the milk fat content over time. There was a sharp decline in the control group between week 4 and week 6. After week 6 there was a steady increase up to week 8 where the protein content normalized again. The decrease in protein content of the control group after week 4 cannot be explained.

The effect of CLA supplementation on lactose content is presented in Figure 5.4.
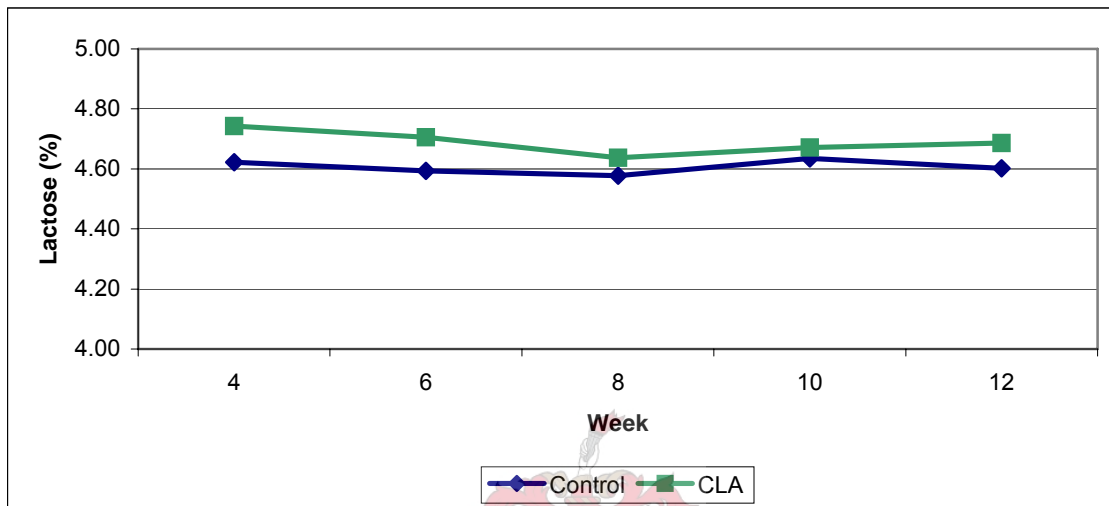
Figure 5.4 The effect of CLA supplementation to pasture-based Jersey cows on weekly mean milk lactose percentage (over 12 weeks).

The lactose content of both treatment groups followed a similar trend from week 4 to week 12.

Treatment effects on the MUN content of milk are presented in Figure 5.5. It is evident that treatment had no effect on MUN levels and that the groups showed a similar increase, followed by a decrease over time. The increase in MUN from week 4 to week 8 might be due to a change in pasture composition during that time.
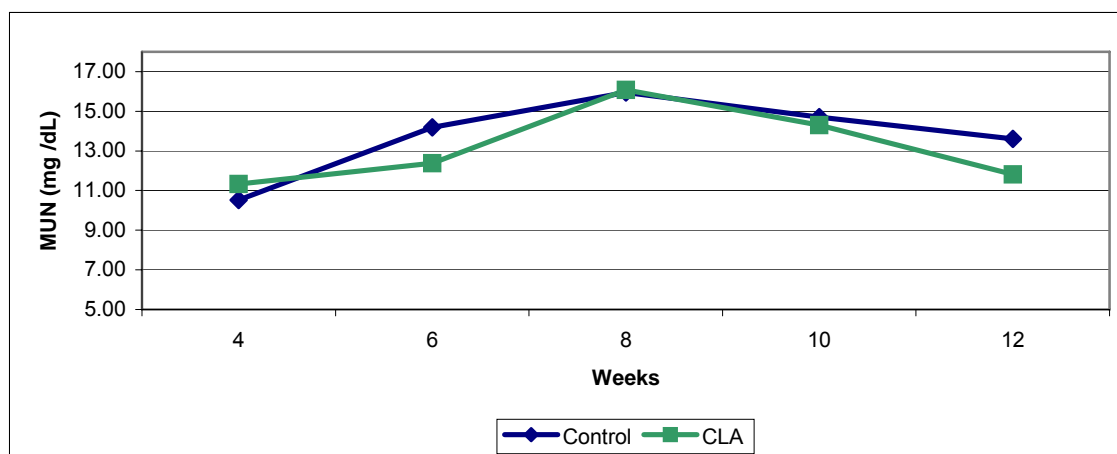
Figure 5.5 The effect of CLA supplementation to pasture-based cows on weekly mean MUN.

Results for BCS and body weight change are presented in Table 5.7. The average body increased by 13.73 and 16.39 kg for the control and CLA groups, respectively, but there was no significant effect caused by the CLA treatment. Supplementing the diet with CLA had an effect on BCS with an average increase in BCS of 0.23 and 0.38 points for control and treatment groups, respectively. This increase in BCS due to the CLA treatment contradicts the findings of Perfield *et al.* (2002). In their study they supplemented lactating cows with Ca salts of CLA FA and the treatment had no effect on BCS.

Table 5.7 Least square means and level of significance for average body condition score (BCS) and body weight.

	Mean ± SEm		P
Treatment	Control	CLA	
BCS	2.23 [a] ± 0.10	2.56 [b] ± 0.10	**0.026**
MASS	376.02 ± 7.46	389.61 ± 7.66	0.211

[a,b] Means in the same row with different superscripts differ ($P \leq 0.05$)

The effect of CLA supplementation on body weight change over time is shown in Figure 5.6, and that on BCS in Figure 5.7.
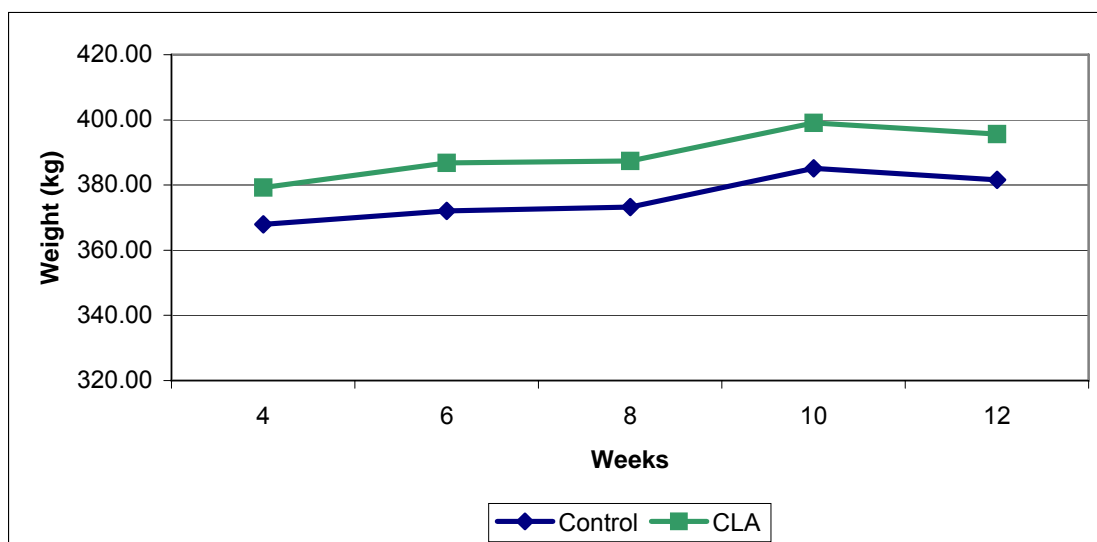
Figure 5.6 The effect of CLA supplementation to pasture-based Jersey cows on mean body weight (over 12 weeks).

From Figures 5.6 and 5.7 it is clear that both groups showed a gradual increase in body weight and BCS. These increases can be explained by the decline in milk production as lactation proceeds leaving the cow with a more positive energy balance. The contribution of foetal weight towards the end of the lactation period may also have impacted on the body weight.
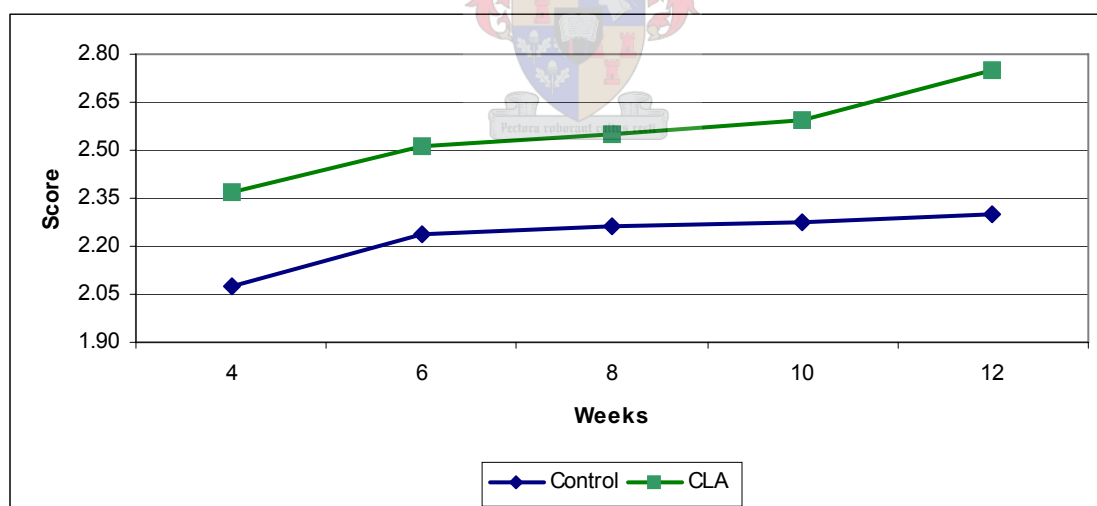
Figure 5.7 The effect of CLA supplementation to pasture-based Jersey cows on BCS (over 12 weeks).

Dietary supplementation of a pasture concentrate with CLA increased the proportion of long chain FA in the milk fat during the first and second month of the trial (Table 5.8). During the 3rd month, there was no difference between treatments in the long chain fatty acid content of milk fat. The proportions of short chain FA were reduced for all three months and medium chain FA were reduced during the first, second and third months. There was an increase in VA content in the second month for the CLA

treatment group. In the CLA treatment, the RA content in milk fat was higher in all three months, with mean values of 2.66, 4.94 and 5.01 mg/g FA for the Control and 3.85, 6.86 and 6.07 mg/g FA for the CLA group. The so-called undesirable saturated FA, $C_{14:0}$ and $C_{16:0}$, were reduced ($P < 0.05$) over all three months in the CLA treatment. Even though the RA content of the CLA diet was significantly increased during this trial, the values were much lower than values from a previous unpublished study in the Western Cape (Chapter 4), where the average of 6 bulk tank samples of Jersey herds on pasture averaged 10 mg/g of FA, and that of 6 Holstein herds under the same conditions with an average of 10.7 mg/g RA. Values of this earlier study was very high, considering that the study was done in late February and March, when pastures are not as lush as in September to December, when the current study was done. It is possible that in this investigation, the copper (Cu) content of the commercial concentrate diet, which was fed to the control as well as to the CLA treatment group, had a depressing effect on CLA synthesis. Cows had an average intake of 240 mg of Cu/day, concentrate and pasture combined, which is higher than the low Cu diets (140 mg/day) that Molares *et al* (2000b) used in their trial. The exact mechanism involved here is not known, but on diets with low levels of Cu cows produced milk fat with higher levels of CLA (Molares *et al.*, 2000a,b).

The desaturase index (Kelsey *et al.*, 2003) was calculated for: *cis*-9 $C_{14:1}$, *cis*-9 $C_{16:1}$, *cis*-9 $C_{18:1}$ and RA and is also presented in Table 5.8. There was an increase in Δ^9 desaturase activity in the second and third month for *cis*-9 $C_{16:1}$, in the second month for *cis*-9 $C_{18:1}$ and in the first and third month for RA. Cows normally rate the same for RA production over a long period of time, even if the diet is changed (Kelly *et al.*, 1998b; Lawless *et al.*, 1999; Peterson *et al.*, 2002).

In the current study, the the cows were rated according to their desaturase activity under the ten highest and ten lowest desaturase indices, out of 40 cows over the three month period, to see if cows fed CLA supplement had higher Δ^9 desaturase activity. The cows that appeared two or three times out of three for a specific index were then listed. The results are presented in Table 5.9. Cows 1-20 were in the control group and numbers 40-60 in the CLA treatment group. From the distribution of the cows in the table, it is evident that 15 of the cows in the control group listed in the ten lowest desaturase indices over the three months. Eight of the control cows listed two or three times with the ten highest indices. In the CLA treatment group, 10 cows appeared in the lowest indices and 10 cows in the highest indices. Cow number two rated consistently three out of three times over all four indices under the 10 lowest indices and cow number 57 rated 2 x 3 and 2 x 2 out of four in the 10 highest desaturase indices. Ten cows in the control group rated in the lowest RA index, and none in the highest, as opposed to the CLA treatment group where 7 cows rated in the 10 highest for the RA index and none in the lowest indices. Therefore, it appears to be reasonable to conclude that CLA treatment may have had a positive effect on the desaturase activity of the lactating cows.

Table 5.8 The effect of CLA supplementation to pasture-based Jersey cows on fatty acid composition of milk.

Fatty acid	Month 1 Control	CLA		Month 2 Control	CLA		Month 3 Control	CLA	
	Mean ± SEM		p	Mean ± SEM		p	Mean ± SEM		p
$C_{10:0}$	$29.52^a \pm 1.0$	$23.9^b 1 \pm 1.0$	**0.000**	$29.15^a \pm 0.9$	$21.12^b \pm 0.8$	**0.000**	$22.71^a \pm 0.9$	$16.56^b \pm 1.2$	**0.000**
$C_{12:0}$	$35.01^a \pm 0.12$	$29.11^b \pm 0.12$	**0.001**	$31.40^a \pm 0.9$	$23.73^b \pm 0.1$	**0.000**	$24.98^a \pm 0.9$	$19.46^b \pm 0.2$	**0.001**
$C_{14:0}$	$112.01^a \pm 2.3$	$101.79^b \pm 2.4$	**0.004**	$103.18^a \pm 2.3$	$89.91^b \pm 2.2$	**0.000**	$88.84^a \pm 1.9$	$73.35^b \pm 2.6$	**0.000**
$C_{14:1 cis}$	5.81 ± 0.4	5.54 ± 0.4	0.619	6.06 ± 0.4	5.49 ± 0.4	0.268	$5.20^a \pm 0.3$	$4.04^b \pm 0.3$	**0.010**
$C_{16:0}$	$330.55^a \pm 5.8$	$307.15^b \pm 6.1$	**0.009**	$297.61^a \pm 5.1$	$270.12^b \pm 5.0$	**0.001**	$277.94^a \pm 4.0$	$262.69^b \pm 5.3$	**0.030**
$C_{16:1 cis-9}$	7.87 ± 0.3	7.47 ± 0.3	0.364	8.59 ± 0.3	8.17 ± 0.3	0.380	$8.15^a \pm 0.3$	$7.13^b \pm 0.4$	**0.033**
$C_{17:0}$	$0.12^a \pm 0.0$	$0.22^b \pm 0.0$	**0.050**	$0.76^a \pm 0.0$	$0.58^b \pm 0.0$	**0.006**	0.59 ± 0.0	0.51 ± 0.0	0.117
$C_{17:1cis}$	$1.08^a \pm 0.1$	$1.86^b \pm 0.2$	**0.019**	1.15 ± 0.1	1.25 ± 0.1	0.335	1.15 ± 0.1	0.97 ± 0.1	0.209
$C_{18:0}$	133.98 ± 0.9	92.75 ± 18.5	0.140	$128.43^a \pm 3.5$	$144.90^b \pm 34$	**0.002**	110.53 ± 5.5	103.13 ± 7.3	0.424
$C_{18:1trans-9}$	1.32 ± 0.1	1.74 ± 0.3	0.234	$3.12^a \pm 0.3$	$5.53^b \pm 0.3$	**0.000**	3.15 ± 0.3	3.21 ± 0.4	0.908
$C_{18:1 trans-11}$	11.30 ± 1.1	18.43 ± 2.2	0.061	$15.45^a \pm 1.0$	$20.82^b \pm 1.0$	**0.001**	16.34 ± 0.8	16.03 ± 0.9	0.786
$C_{18:1 cis-9}$	158.34 ± 5.6	162.32 ± 11.2	0.772	$169.71^a \pm 4.7$	$211.79^b \pm 4.8$	**0.000**	165.73 ± 4.3	176.65 ± 4.7	0.094
$C_{18:1 cis-11}$	5.79 ± 0.5	7.47 ± 1.0	0.212	2.78 ± 0.1	2.70 ± 0.1	0.641	2.60 ± 0.1	2.54 ± 0.1	0.724
$C_{18:3 n-6}$	$0.13^a \pm 0.0$	$0.32^b \pm 0.0$	**0.001**	0.37 ± 0.1	0.40 ± 0.1	0.694	0.40 ± 0.1	0.38 ± 0.1	0.862
$C_{18:3 n-3}$	5.52 ± 0.3	6.08 ± 0.2	0.099	11.81 ± 0.8	12.58 ± 0.6	0.465	12.34 ± 0.4	13.53 ± 0.7	0.171
$C_{18:2 cis 9, trans 11}$	$2.66^a \pm 0.3$	$3.85^b \pm 0.2$	**0.005**	$4.94^a \pm 0.4$	$6.86^b \pm 0.3$	**0.001**	$5.01^a \pm 0.3$	$6.07^b \pm 0.3$	**0.006**
$C_{18:2 trans-10, cis 12}$	$0.37^a \pm 0.1$	$0.71^b \pm 0.1$	**0.003**	$0.41^a \pm 0.1$	$1.12^b \pm 0.1$	**0.000**	$0.32^a \pm 0.0$	$0.98^b \pm 0.1$	**0.000**
Others [1]	57.5 ± 1.7	62.1 ± 1.9	0.080	39.1 ± 11.5	62.9 ± 12.5	0.170	$142.1^a \pm 9.6$	$195.9^b \pm 1.5$	**0.001**
Short [2]	$128.8^a \pm 2.6$	$113.9^b \pm 2.8$	**0.000**	$168.0^a \pm 3.1$	$125.9^b \pm 3.4$	**0.000**	$133.1^a \pm 2.8$	$114.4^b \pm 3.0$	**0.000**
Medium [3]	$467.4^a \pm 7.7$	$436.7^b \pm 8.4$	**0.010**	$434.2^a \pm 5.9$	$388.8^b \pm 6.5$	**0.000**	$389.9^a \pm 6.1$	$367.1^b \pm 6.7$	**0.016**
Long [4]	$346.2^a \pm 9.2$	$387.3^b \pm 10.0$	**0.000**	$358.7^a \pm 8.0$	$422.4^b \pm 8.7$	**0.000**	334.9 ± 9.4	322.7 ± 10.3	0.384
Desaturase index									
$C_{14:1}$	0.049 ± 0.002	0.048 ± 0.003	0.778	0.057 ± 0.003	0.575 ± 0.006	0.986	0.056 ± 0.003	0.604 ± 0.016	0.253
$C_{16:1}$	0.023 ± 0.000	0.023 ± 0.001	0.553	$0.057^a \pm 0.004$	$0.600^b \pm 0.007$	**0.011**	$0.051^a \pm 0.003$	$0.636^b \pm 0.020$	**0.058**
$C_{18:1}$	0.552 ± 0.013	0.532 ± 0.016	0.321	$0.028^a \pm 0.001$	$0.236^b \pm 0.006$	**0.025**	0.029 ± 0.001	0.234 ± 0.005	0.215
RA	$0.187^a \pm 0.004$	$0.230^b \pm 0.005$	**0.000**	0.030 ± 0.001	0.257 ± 0.007	0.253	$0.026^a \pm 0.001$	$0.267^b \pm 0.006$	**0.000**

[a,b] Means in the same row with different superscripts differ (P≤ 0.05)

[1] Sum of unidentified FA

[2] $< C_{16:0}$

[3] $C_{16:0} > \& < C_{18:0}$

[4] $> C_{18:0}$

Table 5.9 Desaturase index for cows who rated two or three times (based on least square means) under the 10 highest or 10 lowest desaturase activity index over the three months.

Cow number	Lowest activity over 3 months				Highest activity over 3 months			
	$C_{14:1}$	$C_{16:1}$	$C_{18:1}$	RA	$C_{14:1}$	$C_{16:1}$	$C_{18:1}$	RA
1	3		3					
2	3	3	3	3				
3	3			2		2		
4	2		2	3				
5				2				
6	3		2	2				
7	3			2				
8	2							
9					3	2		
11							2	
12		2						
13		2		2				
14			2					
15				2	2			
16	2							
17			2	2	2			
18					2			
19		2	2	3				
20					2			
41							3	2
42		2						
43						2		2
45								2
47							2	
48	3							
49	3							
51							2	
52			2					
53								2
54		2						2
55	2	2	2					
56		2						
57					3	2	3	2
58		2					2	2
59	2							
60			2				2	

5.4 Conclusions

This study demonstrated that it is possible to enhance the milk CLA content, and more specifically the RA content, of cows on South African pastures by supplementing their concentrate with a CLA supplement. This supplementation had no effect on the milk yield, protein, lactose or MUN content of the milk, but did cause MFD. The RA content was increased 1.2 – 1.4 fold over that of the control diet. This increase may partially be ascribed to an apparent increase in desaturase activity affected by the CLA supplement. The CLA content of milk was somewhat lower than literature values in both the control and CLA treatments, which could have been due to relatively high levels of Cu in the concentrate. This is, however, speculative and will have to be confirmed by further research.

5.5 References

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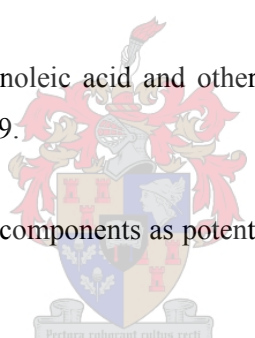
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CHAPTER 6

Effects of tuna oil and Conjugated Linoleic Acid (CLA) supplements in dairy cow diets on CLA levels in milk

Abstract

The objective of the study was to determine responses of an enhanced supply of PUFA and CLA, or a combination of PUFA and CLA, on milk fatty acid composition, with emphasis on milk CLA. Twelve multiparous Holstein cows were used in a 4 x 4 Latin square (n = 3) design. The four diets consisted either of a basal control diet, a basal diet + 1% tuna oil, basal diet + 1% of a CLA supplement, or the basal diet + 0.5% tuna oil + 0.5% CLA supplement. Treatment had no effect on milk production, dry matter intake, as well as protein, lactose and MUN contents of the milk did not differ between treatments. Tuna oil in the diet (1%) decreased milk fat content, with values being 3.21%, 2.84%, 2.94%, and 2.90% for the control, tuna oil, CLA supplement, and tuna oils + CLA supplement treatments, respectively. This also resulted in lower fat yields. The rumenic acid concentration in milk fat was higher in the CLA treatment than in the combined treatment of tuna oil plus CLA supplement. This study demonstrated that it is possible to enhance the milk CLA content by supplementing dairy cow diets with CLA, tuna oil or both.

6.1 Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of the omega 6 essential fatty acid, linoleic acid (*cis*-9, *cis*-12 $C_{18:2}$). The conjugated double bond is found at positions 7 & 9; 8 & 10; 9 & 11; 10 & 12; 11 & 13; 12 & 14 and can be presented in the following geometric configurations; *cis/cis, cis/trans, trans/cis, trans/trans*. CLA is found predominantly in ruminant meat and milk products with milk containing the highest concentrations (Stanton *et al.*, 1997; Rickert *et al.*, 1999; Ritzenthaler *et al.*, 2001; Aydin *et al.*, 2005). Conjugated linoleic acid is not affected by processes commonly used in the dairy industry, and all milk products are therefore good sources of CLA (Lin *et al.*, 1995). The most common isomer of CLA is *cis*-9,*trans*-11 $C_{18:2}$ and milk fat CLA contains up to 94 % of this isomer. This particular isomer has been given the trivial name; rumenic acid (Kramer *et*

al., 1998a), and is the biologically most active isomer of the CLA group (Dugan *et al*., 2001; Collomb *et al*., 2004; Dhiman *et al*., 2005).

In recent years CLA has been investigated extensively by medical as well as nutritional circles. This started in 1985 with the identification of CLA (isolated from ground beef) as an anticarcinogenic agent by Pariza and since then many more health benefits have been contributed to the activity of CLA. The research, mostly on cell cultures, animal models, as well as some human studies has pointed out that CLA may have benefits such as: antiatherogenic, immunomodulating, growth promoting, and lean body mass-enhancing properties, normalize impaired glucose tolerance in noninsulin-dependent diabetes, modulate food allergic reactions, the reduction in growth of melanoma, leukaemia, mesothelioma, and glioblastoma together with breast, prostate, colon, and ovarian cancer as well as two human hepatoma cell lines (Cook *et al*., 1993; Chin *et al*., 1994; Banni *et al*., 1996; Parodi, 1997, 1999; Ip *et al*., 1999; Pariza *et al*., 2001; Weiss *et al*., 2004a,b; Selberg *et al.,* 2004; Aydin *et al.,* 2005). It is indicated by (Pariza, 1997, 1999) that CLA helps to prevent arteriosclerosis, helps to lower high density lipoprotein while razing plasma low density lipoprotein cholesterol levels (Dugan *et al.,* 2001), and protection against the catabolic affects of being exposed to endotoxins (Cook *et al.*, 1993).

The CLA content of milk varies greatly among different production practices such as pasture vs. TMR (Jahreis *et al*., 1996; Dhiman *et al*., 2005, Current study, Chapter 4), animal breeds (Lawless *et al*., 1999; Whitlock *et al*., 2002; Dhiman *et al*., 2005, Current study, Chapter 4), seasons and individual animals (Kelly *et al*., 1998a; 1998b). CLA is synthesised in the rumen during biohydrogenation of linoleic acid (*cis-9, cis-*12 $C_{18:2}$) and linolenic acid (*cis-9, cis-*11, *cis-*15 $C_{18:3}$). CLA, synthesized from linoleic acid (LA), and vaccenic acid (*trans-*11 $C_{18:1}$), synthesized from LA and linolenic acid (LNA), escapes the rumen if biohydrogenation is incomplete, or inhibited by large amounts of substrate (Harfoot & Hazelwood, 1988; Enser *et al*., 1999; Offer *et al*., 1999; Donovan *et al*., 2000; Griinari *et al*., 2000). Vaccenic acid (VA) in turn provides a substrate for endogenous synthesis of CLA in the tissues, accomplished through the activity of Δ^9 desaturase enzyme.

It is therefore possible to enhance the milk fat content of CLA (Stanton *et al*., 1997; Dhiman *et al*., 1999a; Griinari & Bauman, 1999; Bessa *et al*., 2000), by formulating diets that are high in LA, LNA, and VA (e.g. sunflower oil, soybean oil, cottonseed oil, linseed oil, rapeseed oil, or there respective seeds), as well as fish oils (Borques *et al*., 1997; Dhiman *et al*., 2000; Donovan *et al*., 2000) that contain high levels of PUFA. To limit milk fat depression (MFD) caused by high fat diets, rumen protected fats or crushed oil seeds can be included in the diet.

The crushed oil seeds, though high in fat content, are degraded very slowly, and only small amounts of fats are available to the rumen micro-organisms at any period. This leads to enhanced levels of CLA in the milk fat with minimal MFD (Griinari *et al*., 2000; Johnes *et al*., 2000).

The current study was undertaken to determine effects of a CLA supplement, tuna oil and a combination of the two in a TMR on CLA in milk fat. It has been reported that higher levels of CLA may be attained with use of a combination of ingredients, such as a CLA supplement and fish oil, than with the two separately.

6.3 Materials and methods

The study was conducted at the Welgevallen Experimental Farm of the Stellenbosch University from the end of September to mid-December of 2004. Twelve high yielding multiparous Holstein cows in mid-lactation were stratified according to milk production and then randomly allocated to a 4 x 4 Latin square (n = 3) design. The cows were housed in groups of three in a free-stall barn. Groups were kept intact for the duration of the trial and each group remained in the same pen.

The trial lasted 72 days (four periods of 18 days) and each period consisted of 15-day adaptation, followed by a three-day collection during which milk samples were collected and feed intake recorded. Milk production was recorded daily for the duration of the trial, but the average production of the last seven days in each period was used for trial purposes.

The four treatment diets consisted either of a basal control diet, basal diet + 1% tuna oil, basal diet + 1% CLA supplement or the basal diet + 0.5% tuna oil + 0.5% CLA supplement. The basal diet was formulated with the aid of CPM-Dairy v. 3 to provide sufficient nutrients to maintain milk production. Based on the ingredients used, the calculated CP content would be 160 g/kg DM, NDF 340 g/kg DM, ADF 190 g/kg DM, Cu 8.9 ppm and the estimated ME content 10.7 MJ/kg. The physical composition of the basal diet is in Table 6.1. During each trial period, cows received the appropriate TMR at a rate of 30 kg/cow/day. Feed was divided into two daily portions that were offered after milking. A total of 90 kg was thus allocated per group per day. Feed intake per group was recorded on the last three days for each period to calculate average intake of the group.

Table 6.1 Physical composition of the basal diet (control treatment) that was used to determine the effect of a CLA supplement and tuna oil on the CLA content of Holstein milk.

Ingredient	Inclusion rate (kg) per tonne feed DM[1]
Lucerne hay	34.4
Oat hay	367.0
Maize meal	336.2
Cottonseed oil cake	9.4
Soybean oil cake	49.3
Wheaten bran	135.6
Fish meal	29.3
Urea	6.4
Salt	16.2
Limestone	10.8
Mono calcium phosphate	5.4

[1]The following supplemental ingredients were added to the basal diet to compile the four experimental TMR's: 10 kg Luta CLA 20 P per tonne for the CLA treatment; 10 kg tuna oil per tonne for the tuna oil treatment; 5 kg Luta CLA 20 P and 5 kg tuna oil per tonne for the combination treatment.

Cows were milked twice daily at 05:30 and 16:00 and yields were recorded at each milking. Milk samples (400 ml) were collected at a rate of 10 ml /L of milk produced on the evening of day 17, and morning of day 18. Part (40 ml) of the milk sample was preserved with bronopol-B2 preservative for milk composition analysis, and the other part was frozen at -20°C and then freeze-dried and vacuum sealed at the Department of Animal Sciences, Stellenbosch University for fatty acid (FA) analysis. The preserved samples were analyzed for fat, protein, lactose and milk urea nitrogen by the TAURUS milk analysis laboratory in Irene. Final milk composition was based on weighted a.m. and p.m. milk yields.

Milk samples collected during the first and second period for fatty acid analyses were sent to the Skaggs Nutrition Laboratory at the Utah State University, USA. However, when the rest of the batches (last two periods) were sent, they were returned by USA Immigrations due to restrictions on samples from animal origin that had been installed in the USA in the interim period. The samples therefore had to be analyzed locally and the Department of Animal Sciences at the Stellenbosch University had to set up the correct protocol first before these samples could be analyzed. This caused a long delay in completion of analyses.

Milk fat extraction was according to the method of Hara & Radin (1978). The milk was reconstituted by adding 25 ml of de-ionized distilled water to 3-4 g of freeze dried milk. After thorough mixing, the milk was centrifuged at 2500 rpm (1204 x g) for 20 min. at 4 °C. The fat cake was removed and 20 ml of detergent solution (30 g Triton and 70 g sodium hexametaphosphate in 1000 ml de-ionized distilled water) was added. The mixture was vortexed until the cream dissolved and placed in a boiling water bath (100 ˚C) for 20 min. The oil and flocculent layer was removed, added to another 10 ml of detergent solution, vortexed, and placed back into the water bath for 20 min. Approximately 0.1 g of the fat layer was removed, capped under nitrogen and stored at -20 ˚C until analysed for FA.

Milk FA was transesterified with sodium methoxide according to the method of Christie (1982), with modifications. A volume of 2 ml hexane was added to 40 mg of the extracted fat, followed by 40 µl of methyl acetate. After vortexing the mixture for 30 seconds, 40 µl of the methylation reagent (1.75 ml methanol: 0.4 ml of 5.4 M sodium methylate) was added. The mixture was immediately vortexed for 2 min. and allowed to react for a total time of 10 min. To stop the reaction, 60 µl of termination reagent (1 g oxalic acid/30 ml of diethyl ether) was added. A small amount of calcium chloride was added to each tube and the samples were incubated at room temperature for one hour before centrifuged at 2600 rpm (1400 x g) for 5min at 4 ˚C. The hexane supernatant was removed and used directly for gas chromatographic determination.

Fatty acid methyl esters were analyzed by gas chromatography (Hewlett Packard GC system 6890+ with a flame ionizing detector) using a BPX 70 capillary column (120 m x 0.25 mm i.d. with 0.25 µm film thickness). A programmed run with a total run time of 95 min. was used. Gas chromatograph conditions were: initial temp = 130 °C; 1st ramp = 2.0 °C/min with a final temperature of 140 °C; 2nd ramp = 1.0 °C/min with a final temperature of 220 °C; final time = 10 min. Injector temperature was set at 220 °C. Gas flow rates were: hydrogen = 25 ml/min; and hydrogen carrier gas = 2-4 ml/min. An injector split of 80:1 was used with the flame ionizing detector at 260 °C.

The fatty acid percentage of each fatty acid was calculated by dividing the area under the fatty acid peak by the sum of the areas under the total reported FA. Fatty acids were then converted and expressed as mg/g of FA as is typical for scientific literature.

6.3 Statistical analysis

The data from this study was subjected to a main effects ANOVA with the aid of STATISTICA 7. Main effects were treatment and period. Differences between means were separated with a Bonferroni test and significance was declared at P<0.05.

6.4 Results and discussion

Least square means for milk production (MP), milk composition and DMI for the treatments are in Table 6.2. Treatment had no effect on MP, but the three treatment groups had lower milk fat levels and daily milk yields than the control group. Supplementation of tuna oil and or CLA had no effect on milk protein, lactose or MUN. Dry matter intake was also not affected by treatment

Table 6.2 Effects of control, tuna oil, CLA, and combined tuna oil and CLA treatment diets on milk production and milk composition of Holstein cows on TMR.

	Control	Tuna oil	CLA	Tuna oil + CLA	± SEm	P-value
Milk production kg/d	35.15	34.10	34.49	34.16	1.11	0.904
Fat %	3.21[a]	2.84[b]	2.94[b]	2.90[b]	0.09	**0.022**
Protein %	3.10	3.10	3.09	3.07	0.06	0.985
Lactose %	4.75	4.70	4.68	4.72	0.45	0.715
MUN %	15.96	15.53	15.10	14.83	0.43	0.281
Fat kg/d	1.12[a]	0.96[b]	1.00[b]	0.98[b]	0.04	**0.016**
Protein kg/d	1.09	1.05	1.05	1.04	0.03	0.726
Lactose kg/d	1.67	1.60	0.61	1.61	0.05	0.823
Average DMI per cow	29.06	28.27	28.55	28.6		0.752

[a,b] Means in the same row with different superscripts differ (P≤ 0.05)

Effects of treatment on the FA profile of milk is in Table 6.3. Compared to the control, the supplement treatments resulted in a decrease in the $C_{10:0}$ and $C_{12:0}$ short chain FA, while the $C_{14:0}$ acids were not affected. Regarding the medium chain FA, tuna oil supplementation resulted in a decrease in $C_{16:0}$ concentration compared to the control treatment levels, while the other supplements tended to result in decreased levels. Only the CLA supplement increased $C_{17:0}$ levels. Dietary supplementation of the CLA product and CLA + tuna oil increased the $C_{18:1}$ *trans* fatty acids, but the $C_{18:1}$ *cis* isomers were not affected by treatment.

In this study, the interest was mainly in effects of treatment on RA (*cis* 9, *trans* 11 $C_{18:2}$) content. As can be seen in Table 6.4, the CLA supplement, as well as CLA + tuna oil,

increased RA levels in milk fat, while the tuna oil alone only tended to increase RA values. Higher than expected milk RA contents were found for all the treatments with levels ranging from 5.0 for the control to 8.4 for the CLA treatment group. These values are higher than the RA values found in a study with Jersey cows supplemented with CLA on a pasture diet (Chapter 5) where differences in Cu intake may have had an effect. The daily Cu intake of the Jerseys in the previous trial was 240 mg/day. Although the Cu content of the basal diet in the current trial was calculated to be 8.9 ppm, samples of the experimental basal diet were analysed for Cu which indicated that the actual Cu content was only 5.9 ppm. The mean Cu intake of the Holstein cows in the current trial was therefore 168 mg/day. Jerseys also appear to absorb Cu better than Holsteins and Cu has a depressing effect on RA synthesis in the lactating cow (Molares *et al.*, 2000a; b). It is therefore reasonable to assume that the low Cu content in the diet of the Holsteins could have been responsible for the enhanced RA synthesis.

No additive effect on RA content was observed with the combination of the CLA supplement and tuna oil, which is contradictory to the results of Abu-Ghazaleh *et al.* (2002a) who reported an additive effect when diets were supplemented with fish oil and extruded soybean meal, compared to the two supplements added separately. The response observed with the CLA product was somewhat surprising, as this product contains mainly the *trans*-10, *cis*-12 $C_{:18:2}$ isomer of CLA. It is speculated that the product is not as protected from rumen hydrogenation as originally thought and that it could result in an increase in VA which would result in enhanced endogenous production of RA via desaturase activity.

An increase in the content of RA and the trans-octadecenoic acids occured for tuna oil, CLA and tuna oil + CLA with CLA treatment having the highest trans-octadecenoic FA and RA. This effect could be due to the inhibitory effect supplementation of long chain FA in the diet has on microbial growth. The rate-limiting step in the biohydrogenation pathway is not the formation of CLA but the hydrogenation of the VA (*trans*-11-18:1) to stearic acid (Offer *et al.,* 1999). Values for *trans*-10, *cis* 12 $C_{18:2}$ typically reported on diets high in LNA, LA and PUFA range between 0.1 and 1.5 (Abu-Ghazaleh *et al.*, 2002b; Perfield *et al.*, 2002; Lock & Garnsworthy. 2002). Although there was no difference between treatments in *trans*-10, *cis*-12 $C_{18:2}$ the values were higher than expected, but still within the range reported in the literature.

Table 6.3 The effect of control, tuna oil, CLA, and combined tuna oil and CLA treatment diets on fatty acid composition of Holstein cows on TMR.

	Control	Tuna oil	CLA	Tuna oil + CLA		P-value
Fatty acids	Mean	(mg / g of fatty acids)			± SEM	
$C_{:10:0}$	32.0 [a]	27.9 [b]	26.9b [c]	29.0 [ac]	0.8	**0.000**
$C_{:12:0}$	37.6 [a]	32.6 [b]	30.9 [b]	33.2 [b]	0.9	**0.000**
$C_{:14:0}$	121.6	116.1	115.8	117.5	2.1	0.213
$C_{:14:1 \, cis}$	8.9	8.7	8.7	8.5	3.7	0.850
$C_{:16:0}$	312.1 [a]	287.3 [c]	291.5 [ac]	290.0 [ac]	5.6	**0.014**
$C_{:16:1 \, cis-9}$	11.1	10.7	10.7	10.5	0.4	0.781
$C_{:17:0}$	0.3 [a]	0.5 [a]	0.6 [b]	0.4 [a]	0.1	0.022
$C_{:17:1} cis$	1.8	1.9	2.0	1.9	0.1	0.305
$C_{:18:0}$	102.9	104.0	98.1	101.6	3.2	0.609
$C_{:18:1 trans-9}$	2.3 [a]	2.4 [a]	4.0 [b]	2.9 [b]	0.2	**0.000**
$C_{:18:1 \, trans-11}$ (VA)	11.4 [a]	12.4 [a]	22.5 [b]	20.4 [b]	1.3	**0.000**
$C_{:18:1 \, cis-9}$	167.7	178.7	168.2	166.4	4.8	0.264
$C_{:18:3}$	13.6	15.5	13.8	13.2	0.6	0.050
$C_{18:2 \, cis-9, \, trans-11}$ (RA)	5.0 [a]	5.8 [a]	8.4 [b]	7.0 [c]	0.4	**0.049**
$C_{18:2 \, trans-10, \, cis \, 12}$	0.5	1.0	0.5	0.4	0.2	0.099
$C_{:20:5}$	0.8 [a]	0.9 [ab]	1.5 [b]	1.3 [ab]	0.1	**0.001**
Others [1]	51.2 [a]	49.5 [a]	64.4b [c]	66.4 [c]	3.7	**0.003**
Short[2]	156.1	170.4	150.5	155.7	7.9	0.338
Medium[3]	468.1 [ac]	436.3 [b]	441.2 [cb]	440.2 [cb]	7.4	**0.019**
Long[4]	324.6	343.8	343.9	337.7	5.9	0.093

[a,b] Means in the same row with different superscripts differ (P≤ 0.05)

[1] Sum of unidentified FA

[2] < $C_{:16:0}$

[3] $C_{:16:0}$ > & < $C_{:18:0}$

[4] > $C_{:18:0}$

The desaturase indices for the 12 cows are presented in Table 6.5. From the data it is evident that cows with 'n high desaturase index for one fatty acid pair, normally had a high index for the other fatty acid pairs as well and cows with a low index had an overall low index. Cows 2 and 3 had the lowest desaturase activity and cows 6 and 9 had the highest.

Table 6.4 Mean desaturase index and rank of Holstein cows during trial period**.**

Cow	Desaturase index mean				Rank				Overall rank
	C 14:1	C 16:1	C 18:1	CLA	$C_{14:1}$	$C_{16:1}$	$C_{18:1}$	CLA	
1	0.088	0.037	0.663	0.313	1	4	3	6	3
2	0.049	0.031	0.610	0.260	10	10	9	11	11
3	0.041	0.029	0.552	0.213	12	12	12	12	12
4	0.071	0.040	0.643	0.366	7	2	5	2	4
5	0.055	0.033	0.604	0.274	9	8	10	10	10
6	0.088	0.037	0.675	0.335	2	5	1	4	2
7	0.078	0.032	0.645	0.311	6	9	4	7	7
8	0.084	0.036	0.638	0.308	4	7	7	9	8
9	0.080	0.044	0.669	0.368	5	1	2	1	1
10	0.044	0.029	0.557	0.364	11	11	11	3	9
11	0.066	0.039	0.642	0.309	8	3	6	8	6
12	0.084	0.037	0.614	0.324	3	6	8	5	5

6.5 Conclusions

Results indicated that it is possible to enhance the milk CLA content, and more specifically the RA content, of cows in a TMR management system by supplementing their diet with either a CLA supplement or with tuna oil, or with both. This supplementation doe not affect the milk yield, protein, lactose or MUN content of the milk, but does cause MFD. The RA content was higher in the control diet than expected with a level of 0.5 mg/g of FA. As expected, the CLA and or tuna oil treatment had an increasing effect on RA. The CLA and tuna oil + CLA treatment, but not the tuna oil treatment, had decreased levels of $C_{:16:0}$ and increased levels of trans-11 $C_{:18:1}$. It is possible that the relatively low inclusion of Cu in the diet played a role in the increased synthesis of RA, but more research will have to be conducted to confirm this.

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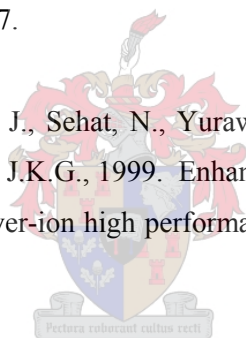
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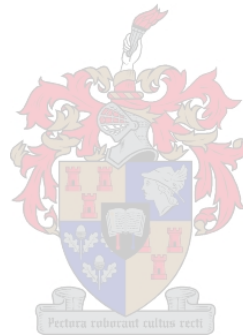
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CHAPTER 7

General conclusions

Concentrations of milk fat, protein and lactose in milk are affected by intake, type of diet, breed of cow, genetic variation, time of year and stage of lactation. The milk fat of dairy cows has unique functional properties and is the constituent of milk solids that can be most readily influenced by nutritional manipulation. The functional component of milk fat that has received a considerable amount of attention in recent years is the group of fatty acids (FA) referred to as conjugated linoleic acid (CLA). The most important of these CLA isomers is the *cis*-9, *trans*-11 $C_{18:2}$ CLA (i.e., Rumenic acid).

The objective of the current study was to increase CLA, and more specifically rumenic acid (RA), in bovine milk fat by nutritional manipulation. Studies attempting to increase the content of CLA in milk fat demonstrated that CLA administration could increase CLA content without affecting milk production, but not without dramatically decreasing milk fat yield. The CLA isomer associated with milk fat depression (MFD) is *trans*-10, *cis*-12 $C_{18:2}$ CLA and this isomer is present in most commercial CLA products and makes up 30 to 50 % of the total CLA isomers in these mixtures. Milk fat depression is also associated with feeding high levels of fat, such as vegetable or fish oils.

Bovine milk samples obtained from bulk tanks in the Western Cape (Chapter 4) indicated that the CLA levels in the Western Cape are well within the range reported in the literature. The difference in CLA levels from milk produced by cows on pasture (10.35 mg/g FA) vs. milk from cows on TMR (4.45 mg/g FA) follows a similar trend as that reported in the literature with higher values from the pasture than from the conserved forages normally used in TMR's.

Results obtained with the CLA supplement offered to pasture-based dairy cows (Chapter 5) indicate that it is possible to substantially increase the rumenic acid and vaccenic acid (VA) contents of milk fat with commercial CLA products. A 1.4 fold increase in the RA content (over 3 months) in milk fat from cows that received the CLA supplement, without affecting milk production, milk yield, protein, lactose or the milk urea nitrogen (MUN) content of the milk was observed. However, this increase in CLA was not attained without causing severe MFD. Treatment had no effect on body mass, but an increase in body condition score occurred in the group receiving the CLA supplement.

Even with the increase of CLA in milk fat due to the CLA supplement, the levels of CLA in the milk fat from Jersey cows on pasture were much lower than expected. The average CLA content of milk fat from cows receiving the CLA supplement was 5.6 mg/g FA compared to an average of 10.4 mg/g FA found in milk produced from pastures in the first (Chapter 4) trial. If taken into consideration that the first survey samples (Chapter 4) were taken in late summer and samples for the second trial (Chapter 5) in early summer, we would still have expected the values of the later trial to be higher.

In the study with tuna oil and CLA supplementation to a TMR (Chapter 6), results were similar to those reported in literature. The CLA levels in the milk fat from Holsteins on all four diets were surprisingly high in comparison with the milk fat from the pasture-based Jersey cows (Chapter 4). The average CLA content of milk fat from TMR-based cows over all the diets was 6.6 mg/g FA compared to the average value of 5.1 mg/g FA observed in milk fat from pasture-based cows. Milk from cows receiving the CLA supplement had the highest levels of RA and VA, followed by the combination of CLA and tuna oil.

In this experiment, the dietary treatment had no effect on milk production or milk yield, protein, lactose or MUN. However, once again MFD was observed with the diets containing tuna oil, CLA supplement and the combination of CLA and tuna oil.

In conclusion, levels of RA and VA in milk fat can be increased by feeding CLA supplements on pasture, as well as feeding CLA supplements with or without tuna oil in TMR feeding systems. These enhanced levels of CLA in milk fat would increase the role of milk as a functional food and has potential to boost milk consumption in the future.