UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

FACULTY OF VETERINARY MEDICINE

Dottorato di ricerca in "Produzione e Sanità degli Alimenti di Origine Animale"
Curriculum in Produzioni Animali
XXIII Ciclo

"Conjugated Linoleic Acid and dairy cows: metabolism, reproduction and products quality"

Dissertation submitted for the degree of Philosophie Doctor
December, 2010

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Dal più antico degli argomenti
trarremo la più nuova delle scienze.

Herman Ebbinghaus

Alla famiglia (la mia),
all'autoironia,
alla Vita e alla libertà di pensiero,
a Ithaca non terra d'arrivo bensì di partenza…

Acknowledgments:

- My sister Flavia, good and faithful assistant and friend in all these years.

- Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali Università degli Studi di Napoli Federico II.
  In particular my Advisor Prof Antonio Di Francia and my tutor Dott. Felicia Masucci , with which I enjoyed and shared two years in Portici,
  Dott. Maria Luisa Varricchio my colleague and friend, lab assistant Mr. Alfredo Maiolino also called "Renegade".

- Department of Animal Science, Cornell University, Ithaca New York, in particular Chairman and Prof. W. Ronald Butler that gave me the big opportunity to "live" Cornell, to think and to believe in me as a scientist.
  Prof. Robert O. Gilbert that taught me a lot but specially to believe in me as a Vet.
  Lab assistant Mrs. Susanne H. Pelton , untiring worker, always happy, full of energies and really important for me and my experiment.
  Emiliano Raffrenato, that always supported me morally and practically for all the year and with I enjoyed all the time in USA.
  Victor Absalon Medina and Augusto Schneider, my colleagues and friends.
  Patricia Marie Dalglish, faithful assistant in the barn and great friend
  Takashi Yasui and his wife for his help and their friendship.

- Dott. Corrado Pacelli, the spark.

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Chapter 1

Aims

There is considerable support among the nutritional communities for the diet-heart (lipid) hypothesis, the idea that an imbalance of dietary cholesterol and fats are the primary of atherosclerosis and cardiovascular disease (CVD) (Griel and Kris-Etherton, 2006). Health professionals world-wide recommend a reduction in the overall consumption of satured fatty acids (SFA), trans-fatty acids (TA) and cholesterol, while emphasizing the need to increase intake of n-6 and n-3 polynsatured fats (Griel and Kris-Etherton, 2006;Kris-Etherton et al., 2007).

Such broad sweeping nutritional recommendations with regard to fat consumption are largely due to epidemiologic studies showing strong positive correlations between intake of SFA and incidence of CVD, a condition believed to result from the concomitant rise in serum low-density-lipoprotein (LDL) cholesterol as SFA intake increase (Hu et al., 1997; Posner et al., 1991). For example, it is generally accepted that for every 1% increase in energy from SFA, LDL cholesterol levels reportedly increase by 1.3 to 1.7mg/dL (0,034 to 0,044 mmol/L) (Mensink and Katan, 1992; Mensink et al., 2003).

Wide promotion of this correlative data spurred an anti-SFA campaign that reduced consumption of dietary fats, including most animal proteins such as meat, dairy products and eggs over the last 3 decades (Putnam et al., 2002), indicted on their relatively high SFA and cholesterol content.However, more recent lipid research would suggest that not all SFAs have the same impact on serum cholesterol.For istance, lauric acid (C12:0) and myristic acid (C14:0) have a greater total cholesterol raising effect than palmitic acid (C16:0), whereas stearic acid (C18:0) has a neutral effect on the concentration of total serum cholesterol, including no apparent impact on either LDL or HDL. Furtheremore, the monunsatured Fatty Acids (MUFA), specially palmitoleic acid (C16:1) and oleic acid (C18:1) are able to lower the level of the LDL improving the ratio LDL/HDL lowering the cholesterolemy.

From the polyunsatured fatty acids, there are two essential fatty acids (EFAs) in human nutrition: a-linolenic acid (aLA), an omega-3 fatty acid; and linoleic acid (LA), an omega-6 fatty acid. The human body cannot synthesize essential fatty acids, yet they are critical to human health; for this reason, EFAs must be obtained from food. Both aLA and LA serve as precursors of other important compounds. For instance, aLA is the precursor for the omega-3 pathway. Likewise, LA is the parent fatty acid in the omega-6 pathway. Omega-3 (n-3) and omega-6 (n-6) fatty acids are two separate distinct families, yet they are synthesized by some of the same enzymes; specifically, delta-5-desaturase and delta-6- desaturase.

Milk and dairy products are recognized as important sources of nutrients in human diets, providing energy, high quality protein, and essential minerals and vitamins (Etherton, 1988; Siekmann et al.,). Milk fat is responsible for many of the sensory, physical, and manufacturing properties of dairy products (Kaylegian and Lindsay, 1995). However,

milk fat is relatively more saturated than most plant oils, and this has led to a negative consumer perception and a public health concern related to excessive intake of saturated fats. Milk fat content and FA composition can be significantly altered through nutrition of the dairy cows, offering the opportunity to respond to market forces and human health recommendations (Lock et al., 2004). The impact of dairy cow nutrition on fat content and FA composition of milk has been extensively reviewed (Lock et al., 2004; (Sutton, 1989). Due to increased consumer awareness of the link between diet and health, research has focused on altering the FA composition of cows' milk to achieve a FA profile consistent with consumer perceptions and health recommendations.

One of the most important omega-6 fatty acid is the Conjugated Linoleic Acid, a mixture of geometric and positional isomers of linoleic acid with conjugated double bonds. CLA is found predominately in food products from ruminant animals; milk and other dairy products are the major sources of CLA in the human diet (Lp et al., 1994; Parodi, 1994; Lp et al., 2006b). Furthermore, the cis9-trans 11 isomer is the major CLA isomer found in milk fat and is thought to be a active form that possesses anticarcinogenic capabilities (Belury, 1995; Parodi, 1997; Lp et al., 2006a; Lp et al., 2006a). Typical concentrations of CLA in milk fat are 3–6 g/kg fat, but CLA concentration in milk can vary widely among herds (Riel, 1963), which may be a consequence of dietary differences. However, the specific factors that cause these variances have not been extensively investigated. Griinari & Bauman (Griinari et al., 1999) proposed that dietary factors which affect milk CLA content could be grouped into one of two categories. The first would be factors that provide lipid substrates for formation of CLA or *trans*-18:1 in the rumen. The second would be factors that change the microbial activity associated with rumen biohydrogenation. Plant oils high in linoleic acid (e.g.sunflower, soyabean and rapeseed) are very efficient at increasing milk CLA content. Besides increasing the yield of CLA and *trans*-18:1 directly, it is likely that linoleic acid inhibits the final reduction of *trans*-18:1, thus increasing its accumulation in the rumen (Griinari & Bauman, 1999).The CLA content of milk and milk products can be altered by affecting rumen production of CLA or *trans*-11-18:1, or by dietary supplementation with these fatty acids (Chouinard et al., 1999). Indeed, Jiang *et al*. (Jiang et al., 1996) reported a variation of 0·25–1·77 g CLA/100 g milk fatty acids and suggested that there is scope for increasing the CLA content of milk through changes to the cows' diet. Milk fat from pasture fed cows seems to be higher in linolenic acid than milk fat from cows receiving preserved grass or maize, but the magnitude of this difference is limited. Little information is available about the effect of the nature of forage on milk fat composition. Indeed, the modulation of milk fat composition is generally achieved by lipid supplements, and not by the choice of the forage. There are very few direct comparisons allowing to evaluate precisely the effects of basal forage diet on milk FA composition.

However, indirect comparisons suggest that milk fat from maize silage diets is richer in short chain FA and linoleic acid when compared to that obtained from grass silage diets. Compared to fresh grass, grass silage favours myristic and palmitic acids at the expense of mono- and polyunsaturated FA, including CLA.(Chilliard et al., 2000)

Furthermore in dairy cow at parturition copious milk synthesis result in abrupt increase in nutritional requirements, and this, along with a gradual decrease of dry matter intake (DMI), leads to negative energy balance (NEBAL), which persist during early

lactation. The severity and extent of NEBAL in early lactation tends to be related to DMI more than to milk production, and it reduces postparun luteinizing hormone (LH) pulsatility delaying first ovulation postpartum (reviewed by (Butler, 2000). Therefore nutritional management during the prepartum period and early lactation is important to shorten the extent of NEBAL, to manage body condition score losses, and to optimize reproduction.

Utilization of fat in dairy rations has become a common practice to increase energy density of the diet. Fat may also positively influence reproductive variables by proving precursors for steroid hormones. In addition, there is some evidence that unsaturated fatty acids may modify uterine release of Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) improving embryo survival (Staples et al., 1998; Mattos et al., 2000). Unsaturated long chain fatty acids can also act as signaling molecules that regulate gene expression (Jump, 2004).

So, these points had opened a new window of opportunity to improve reproductive efficiency and milk's product quality through fat supplementation.

The present dissertation involves a series of experiments which have the objective of assessing the effect of fat supplementation, specifically CLA's isomers, on the quality of dairy products and on fertility and metabolism of dairy cows.

The first experiment evaluated the effect of pasture allowance on FAs composition and sensorial proprieties of milk and cheese

The second study evaluated in dairy cows the effects of supplying CLA from the peripartum period until the breeding period on metabolism and fertility analyzing the related markers.

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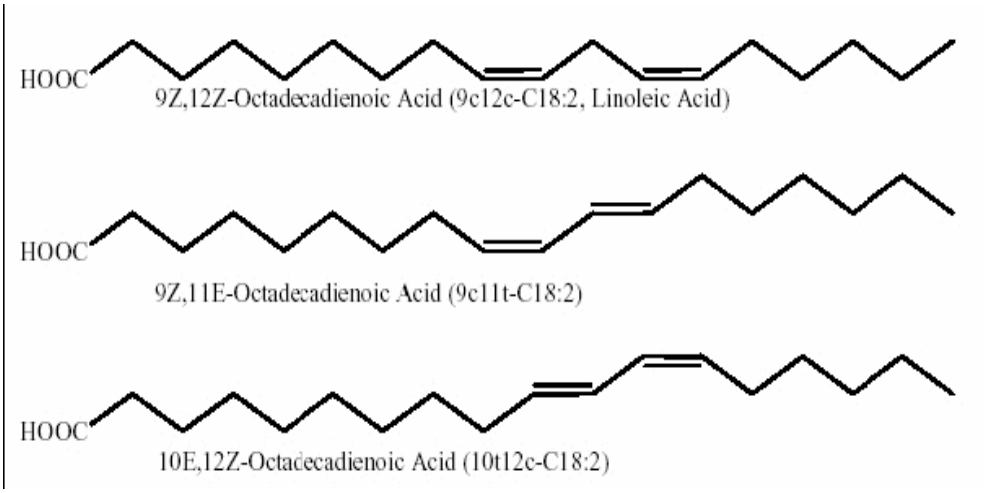
Chapter 2

Literature Review:

2.1. Introduction

CLA is a collective term describing a mixture of positional and geometric isomers of linoleic acid (fatty acid with eighteen C) with conjugated double bonds separated by one single bond (M.A. McGuire; M.K. McGuire; M.S. McGuire and J.M. Griinari, unpublished results). The two unsaturated double bonds in CLA are usually either the C atoms in positions 9 and 11 or at positions 10 and 12 (from thecarboxylic end); however there are other possibilities (figure 2.1). At each double bond position, it is possible for the H atoms to be in either the *cis* or *trans* configuration (in figure 1 the two most common CLA's structure: *cis* 9- *trans*-11 and *trans*-10 cis-12). The *cis*-9,*trans*-11-CLA isomer is believed to be the active form because only this isomer is incorporated into the phospholipids fraction of tissues of animals fed a mixture of CLA isomers (Ha et al., 1990). Recently, there has been a surge of interest in CLA in man's diet because of increasing evidence, based largely on animal studies, suggesting potential benefits of CLA for man‘s health (Lp et al., 1994). Although CLA occurs naturally in many foodstuffs, the principal dietary sources are dairy products and other foods derived from ruminant animals (Chin et al., 1992).

Figure 2.1: Chemical structure of linoleic acid and CLA



2.1.1Dietary sources of conjugated linoleic acid for man

Dairy products

Table 2.1 shows the CLA concentration in different foods and Table 2.2 indicates the major sources of CLA. Milk fat has the greatest potential for high CLA and certainly elevated concentrations of *cis*-9,*trans*-11 isomer. MA McGuire, MK McGuire, MS McGuire and JM Griinari (unpublished results) showed that human milk is devoid of CLA when ruminant products are removed from their diet. Fig. 2.2 shows that the CLA content in milk fat is greatest for ruminant animals, particularly cows and sheep. The primary isomer of CLA, *cis*-9,*trans*-11-octadecadienoic acid accounts for more than 82·0 g/100 g total CLA isomers in dairy products (Chin *et al*. 1992). However, the CLA content in milk and cheeses varies considerably, ranging from approximately 3 to 9 g/kg fat (Chin et al., 1992). Dhiman *et al*.(Dhiman et al., 1999b) reported CLA contents in samples of milk of 3·4, 6·9 and 6·0 g/kg milk fat from cows offered diets containing 135 g soybean meal/kg, 120 g full-fat soybeans/kg or 120 g full-fat cotton seed/kg respectively and reported no change in CLA content in mozzarella cheese processed from the same milk.

Table 21: Total conjugated linoleic acid and *cis*-9,*trans*-11-conjugated linoleic acid in different food products*

Food	Total CLA (g/kg fat)	*cis*-9,*trans*-11 CLA (g/100 kg total CLA)
Butter	9·4–11·9	91·0
Processed cheese	3·2–8·9	17·0–90·0
Natural cheese	0·6–7·1	17·0–90·0
Yoghurt	5·1–9·0	82·0
T-bone steak (cooked)	4·7–9·9	65·0
T-bone steak (raw)	4·4–6·6	59·0
Vegetable oils	0·2	45·0
Milk fat	2·0–30·0	90·0

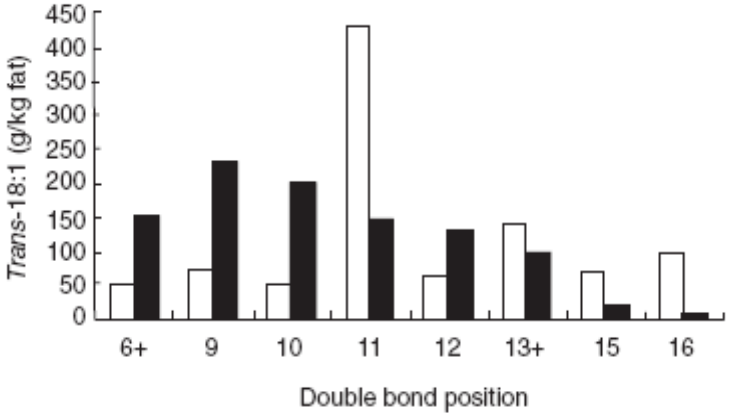
CLA, conjugated linoleic acid.
*Adapted from O'Shea *et al.* (1998) and Chin *et al.* (1992).

Table 2.2: The contribution of various components of the UK diet to conjugated linoleic acid intake*

Food source	Assumed average fat intake from the food source (g/d)	CLA intake by man (mg CLA/d)
Meat	17·5	49·00
Fish	1·3	0·55
Cheese	4·6	25·30
Butter	4·5	27·50
Milk	8·5	53·00
Vegetable, salad oils and margarine	9·3	1·90

CLA, conjugated linoleic acid.
*Adapted from Ministry of Agriculture, Fisheries and Food (1995) and Chin *et al.* (1992).

Fig. 2.2: Distribution of *trans*-18:1 isomers in milk fat (□), and hydrogenated vegetable oil (■) where cows were offered various different feeds. Adapted from Precht & Molkentin (1995). The hydrogenated vegetable oil was margarine and cooking oil.



2.1.2 Relationship with trans fatty acids

Rumen biohydrogenation results in a characteristic pattern of positional isomers of *trans*-18:1 where *trans*-11 is the major isomer comprising at least 80·0 g/100 g *trans*-18:1 (Kemp et al., 1984). This pattern is reflected in the tissue lipids and milk fat of ruminants (Wolff, 1995). Chemical hydrogenation of polyunsaturated fatty acids produces a distinctly different pattern of positional *trans* isomers, where the proportions of isomers with *trans* double bonds in positions 9, 10 and 11 are almost equal (Fig. 2.3). The increased concentration of both *trans*-18:1 and CLA in milk fat compared with hydrogenated vegetable oil could result from similar proportional inhibition of both enzymes required for production of *trans*-18:1 and CLA by the dietary *n*-3 polyunsaturated fatty acids or feedback inhibition of CLA reductase by increased concentrations of *trans*-18:1 in the rumen (Griinari et al., 1996). Enser *et al*.(Enser et al., 1999) reported a strong linear correlation between CLA and *trans*-18:1 concentrations in beef cows. A similar relationship between these two fatty acids has been reported in milk indicating that the effect stems from the rumen rather than specific metabolism in the mammary gland. Furthermore, this correlation could reflect the desaturation of vaccenic acid by Δ9-desaturase.

In summary, there is scope for increasing CLA concentrations in milk by feeding to affect rumen production of CLA. This may include pasture management, concentrate formulation and techniques for protection of plant oils.

Fig. 2.3: Conjugated linoleic acid (CLA) content in milk fat of different species. Adapted from Jahreis *et al* (Jahreis et al., 1999).

2.1.3 Dietary fat in ruminants

The diet of ruminants typically contains about 5% fat and the general recommendation is that fat should not exceed 7% of Dry Matter (Doreau and Chilliard, 1997). Fat that is present in diet feed stuffs is commonly in the form of glycolipids (forages) and triglycerides (concentrates). The most abundant fatty acids present in feedstuff are linoleic and linolenic acid (Harfoot et al., 1988a; Harfoot et al., 1988b). Feedstuff has, in average, the 3,5% of total fat and from this, fatty acids (FA) are the 40% of the total fat in the forages and 70% in the concentrates. In the temperate zones, forages has about 1-3% of FA (most of these is α-LA). In the tropical zones, instead the percentage of α-linolenic acid out of the total FA is from 15% to 40% (Chilliard et al., 2000).

Anyway, the FA's acidic composition change based on the species of forages and on the use of the pasture (Dewhurst et al., 2001).

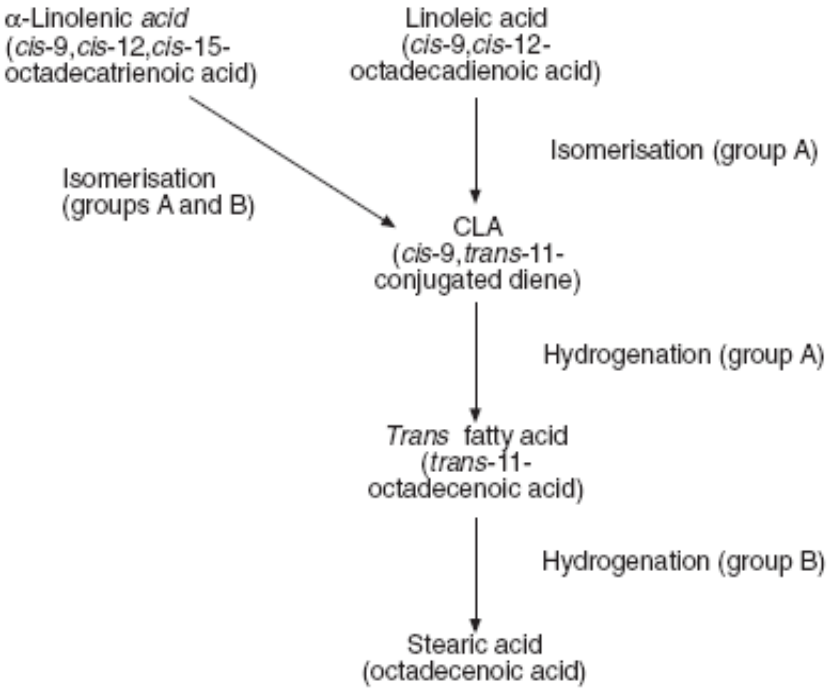
2.1.4 Synthesis by rumen bacteria

The rumen is the site of intense microbial lipid metabolism. Lipolysis of dietary glycolipids, phospholipids and triacylglycerol releases free fatty acids, which are hydrogenated to a large extent (Harfoot and Hazlewood, 1997). The amount of conjugated dienoic acids in cows' milk(Bartlet and Chapman, 1961) and butter (Parodi, 1977) has been correlated positively with dietary intake of linoleic acid, indicating that CLA formed in the rumen is incorporated into milk fat (Bartlet and Chapman, 1961; Parodi, 1977).

Kepler identified the *cis*- 9,*trans*-11-CLA isomer as an intermediate in the biohydrogenation of linoleic acid by the rumen micro-organism *Butyrivibrio fibrisolvens* (Kepler et al., 1966). In a review, Viviani (1967) (Viviani, 1967) proposed that CLA was also formed as an intermediate in the biohydrogenation pathway of linoleic acid. However, in the biohydrogenation studies with rumen micro-organisms, α-linolenic acid (*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid) has been showed to be converted to *cis*-9,*trans*-11,*cis*-15-conjugated triene, then to *trans*-11,*cis*-15-18:2, and finally to an octadecenoic acid which is either *trans*-11, *trans*-15, or *cis*-15 (Harfoot et al., 1988b). Therefore, the pathways from α-linolenic acid do not involve CLA as an intermediate. Although linoleate isomerase and CLA reductase have been purified from the bacteria *Butyrivibrio fibrisolvens*(Kepler et al., 1966; Hughes et al., 1982), in general, no one species of micro-organism carries out the full sequence of biohydrogenation (Harfoot et al., 1988b) The extent of rumen biohydrogenation mainly depends on the type of diet. This has been showed to be due to a drop in pH, limiting at first lipolysis, and thus hydrogenation, which occurs only on free fatty acids(Van Nevel and Demeyer, 1996). A large amount of dietary linoleic acid and a decrease in the rate of hydrogenation are the two main factors that contribute to an increase in the concentration of the intermediate compounds CLA

and *trans* monounsaturated fatty acids. The pathway of biohydrogenation of linoleic acid to stearic acid by rumen micro-organisms is showed in Fig. 2.4.

Fig. 2.4: Pathway of biohydrogenation of linoleic and α-linolenic acids to stearic acid by the rumen. CLA, conjugated linoleic acid. Adapted from Harfoot & Hazlewood (1988). Note that group A bacteria mostly hydrogenate linoleic and α-linolenic acids to *trans*-11-octadecenoic acid. Group B bacteria are capable of hydrogenating octadecenoic acid to stearic acid



2.1.4 Control of rumen biohydrogenation

Little information is currently available regarding the biochemical mechanism that regulates the metabolism of the different CLA isomers in the ruminant animals. Changes in substrate supply and extent of biohydrogenation will affect the supply of intermediate and endproducts of biohydrogenation, thus influencing the CLA content of milk from ruminants (Kelly et al., 1998b; Dhiman et al., 1999b). However, it is the penultimate step (the hydrogenation of the *trans* monoene) that is thought to be rate limiting and subject to modification.

Free fatty acids liberated by lipolysis are adsorbed onto particles, where they are both hydrogenated and/or incorporated into the lipid fraction of the solid-associated bacteria (Demeyer and Doreau, 1999). According to Harfoot & Hazlewood (1997), the balance between these processes is one of the factors determining the extent of fatty acid biohydrogenation in the rumen. A range of hydrogenating bacteria has been isolated and can be divided into groups A and B. Group B bacteria are capable of hydrogenating a wide range of octadecenoic acids to stearic acid. Group A hydrogenate linoleic and α-linolenic acids mainly to the *trans*-11-octadecenoic acid or vaccenic acid, with smaller amounts of other positional and stereo-isomers of the same acid. The initial step in the biohydrogenation of linoleic acid involves the isomerisation of the *cis*-9, *cis*-12 isomer to the *cis*-9, *trans*-11 isomer, which is followed by a preferential reduction of the *cis*-9 double bond to form the *trans*-11-18:1. Biohydrogenation only occurs with free fatty acid, but the system is easily overloaded, with inhibition of the process by the free acids and accumulation of *trans*-18:1 and CLA (Fellner et al., 1997). The lipase enzyme responsible for lipid breakdown is inhibited by low pH. This may explain the decreased degree of saturation in rumen and duodenal lipids and/or body fat in animals fed on concentrate diets (Kobayashi et al., 1992). Gerson *et al*. (Gerson et al., 1985) showed that other factors in addition to pH probably relate to changes in microbial populations. In addition to vaccenic acid, *trans*-10-octadecenoic acid is also found in cows' milk (Griinari et al., 1999). Verhulst *et al*. (Verhulst et al., 1985) isolated a micro-organism that converts linoleic acid to *trans*-10,*cis*-12-CLA, so it is likely, by analogy to vaccenic acid, that *trans*-10-octadecenoic acid may form in the rumen via microbial metabolism of linoleic acid to *trans*-10,*cis*- 12-CLA, which is then biohydrogenated at the *cis*-12 bond. Since mammals do not possess α12-desaturase, it follows that the *trans*-10, *cis*-12-CLA reported in ruminant tissues would originate from *trans*-10, *cis*-12-CLA that was absorbed from the gastrointestinal tract. Although the pathway of hydrogenation of linoleic acid in the rumen is well established, the effects of polyunsaturated fatty acid concentration on the individual enzymes are unclear. The isomerase that catalyses the first step in which CLA is produced has been purified from rumen bacteria and found to be inhibited by high concentrations of linoleic and α-linolenic acid (Kepler et al., 1966).

2.1.5 Effects of fat protection on conjugated linoleic acid production in the rumen

Increasing interest in using dietary fat supplements for ruminants, initially to increase energy density of the diet and more recently to allow delivery of unsaturated fatty acids for absorption in the small intestine, has led to development of rumen-protection forms. An ideal rumen-stable delivery system should fully protect the nutrient from fermentation in the rumen and then allow it to be completely released for post-ruminal absorption (Wu and Papas, 1997). Since the 1970s different attempts have been made to protect lipids against biohydrogenation. Although the degree of protection is sometimes uncertain, this technique is to date the only one which results in large amounts of polyunsaturated fatty acids escaping rumen degradation.

When protected fat is offered to dairy cows the CLA content of their milk is reduced. This effect is due to protection of the lipid molecule, resulting in the group A isomerisation bacteria being unable to convert linoleic acid into CLA. Among the techniques that have been investigated, the use of Ca salts is very popular. The ability of Ca salts to prevent interactions between fatty acids and microbes has been demonstrated for palm oil fatty acids (Chilliard et al., 2000). Further investigations are required to produce a method of fat protection that allows fatty acid isomerisation but protects from excessive biohydrogenation.

2.1.6 Absorption and transport

The epithelial cells are the site for esterification of glycerol into triacylglycerols and phospholipids, which are transported into the lymph as chylomicrons and VLDL, which is the main route in ruminants, and further into the blood where these lipoproteins are found together with LDL and HDL. LDL and VLDL deliver most preformed fatty acids to the mammary gland. Although HDL account for approximately 900 g/kg blood lipids they consist largely of phospholipids, cholesterol and cholesteryl esters, containing the major proportion of polyunsaturated fatty acids (Mansbridge and Blake, 1997). Lipoproteins transport fatty acid mainly to the mammary gland in dairy cattle and mainly to adipose and muscle tissue in fattening animals.

When fatty acids are needed for energy production, that is, synthesis of milk fat, VLDL lose most of their triacylglycerol and are converted into LDL and HDL. Chouinard *et al*.(Chouinard et al., 1999) reported that there appears to be some selectivity in the uptake or incorporation of the *cis*-9, *trans*-11 isomer over the *trans*-10 isomer of CLA in dairy cows. There were differences in the efficiency of transfer of CLA to milk fat among the isomers. Only about 10 g/100 g dietary supplement of *cis*-10, *trans*-12-CLA isomer was transferred to milk fat, whereas the *cis*-8,*trans*-10, *cis*-9,*trans*-11- and *cis*-11,*trans*-13-CLA isomers were transferred to milk fat with over twice the efficiency (22·0–26·0 g/100 g; Fig. 2) (Chouinard et al., 1999). Studies in lactating dairy cows have also found that the transfer efficiency of a dietary supplement of the *cis*-10,*trans*-12-CLA isomer was

only about half of that observed for the *cis*-9,*trans*-11-CLA isomer (Chouinard et al., 1999).

2.1.7 Adipose tissue and the mammary gland as conjugated linoleic acid deposit

Baumgard *et al*. (Baumgard et al., 2000) reported that the *trans*-10, *cis*-12-CLA isomer would decrease milk fat synthesis. CLA may be inhibiting the activity or synthesis of key enzymes involved in *de novo* fatty acid synthesis. The specific mechanisms whereby CLA alters lipid metabolism are not clear. One mechanism may involve increases in rates of lipolysis and fatty acid oxidation inadipose tissue. In lactating cows, circulating concentrations of plasma non-esterified fatty acids are highly correlated with rates of lipolysis and the relatively minor changes observed with CLA supplementation suggested that CLA had little or no effect on lipolysis(Bauman et al., 1988). When CLA supplement was infused abomasally to by-pass rumen biohydrogenation, Chouinard *et al*. (1999) observed a dramatic reduction in milk fat of lactating cows, whereas milk yield and protein were unaffected. The addition of the CLA supplement increased the milk fat content of CLA in a dose-dependent manner from approximately 7 mg/g fat at the zero dose to 64 mg/g fat at the high dose of 150 g CLA supplement/d (90 g actual CLA isomers/d).

Another possible mechanism by which CLA might alter lipid metabolism would be to reduce tissue uptake of fatty acids. This involves lipoprotein lipase, and the activity of this enzyme was decreased when 3T3-L1 adipocyte cultures were incubated with CLA (Park et al., 1997). If lipoprotein lipase in the mammary gland was affected in this study then a reduction in the use of preformed fatty acids for milk fat synthesis would be expected. A reduction was observed, but effects on *de novo*-synthesised fatty acids were more extensive.

2.1.8 De novo synthesis

In certain circumstances, fatty acids can be synthesised *de novo* from acetate. Adipose tissue is the major site of fatty acid synthesis in ruminants, except during lactation when the mammary gland becomes the predominant site. Synthesis of fatty acids up to palmitic acid takes place in cytoplasm from acetyl-CoA and β-hydroxybutyrate derived from mitochondrial oxidation.

Mitochondria elongate palmitic acid to longer-chain fatty acids up to C22, whereas microsomes are capable of elongation as well as desaturation of fatty acids $\geq$C18. In the mammary gland, *denovo* synthesis is generally limited to short-chain fatty acids and medium-chain fatty acids (up to C16:0). However, Corl *et al*. (1998) suggested a possible

synthesis of CLA in the mammary gland from *trans*-11-18:1 by the Δ9-desaturase. Offer *et al*. (1999) also suggested that CLA is formed from *trans* monoenes within the animal tissues such as the mammary gland. Griinari & Bauman (1999) suggested that the ruminant mammary glands and the adipose cells are able to synthesise *cis*-9,*trans*-11-CLA from *trans*-11-18:1 and other CLA isomers from other *trans*-18:1 isomers by action of the Δ9-desaturase on *trans*-18:1. Ward *et al*. (1998) showed in sheep that the Δ9-desaturase expression decreased in adipose tissue and increased in mammary tissue with the onset of lactation. Griinari & Bauman (1999) suggested that about 33 g/100 g *trans*-11-18:1 taken up by the mammary gland is desaturated to *cis*-9,*trans*-11-CLA. The presence of *trans*-18:1 in ruminant milk could enhance its value for consumption by man, since rodent tissues can also convert *trans*-18:1 into CLA(Santora et al., 2000). The Δ9-desaturase genes have been identified in tissues from human subjects(Tocher et al., 1998) but the desaturation of 18:0 to 18:1 has not been detected in the mammary gland of human subjects (Jensen, 1999).

2.1.9 Identification of feeding strategies which promote conjugated linoleic acid production in the rumen and secretion in milk

Griinari & Bauman (1999) proposed that dietary factors which affect milk CLA content could be grouped into one of two categories. The first would be factors that provide lipid substrates for formation of CLA or *trans*-18:1 in the rumen. The second would be factors that change the microbial activity associated with rumen biohydrogenation. Typical concentrations of CLA in milk fat are 3–6 g/kg fat, Combinations of these various factors induce wide variations of milk CLA and *trans*-18:1 concentrations (up to 4% rumenic acid and 10% vaccenic acid, Fig. 2.5), and strong interactions occur between forages, starchy concentrates and lipid supplements (tab. 2.3). Griinari & Bauman (1999) showed that plant oils high in linoleic acid (e.g.sunflower, soybean and rapeseed) are very efficient at increasing milk CLA content. Besides increasing the yield of CLA and *trans*-18:1 directly, it is likely that linoleic acid inhibits the final reduction of *trans*-18:1, thus increasing its accumulation in the rumen.

Figure 2.5. Relationship between the contents of trans-vaccenic and rumenic acids in goat milk. Each point is the mean of values from 7 to 16 goats (N = 401 milks from 38 experimental groups) (adapted from [7]). ○ Hay-based diets (either without lipid supplementation or with untreated lupin seeds or soybeans). ● Hay-based diets (either without lipid supplementation or supplemented with high-oleic sunflower oil or untreated linseeds or sunflower seeds) or corn silage-based diets (either without lipid supplementation or with high-oleic sunflower oil). ■ Hay or corn silage-based diets, supplemented with either linseed oil or sunflower oil

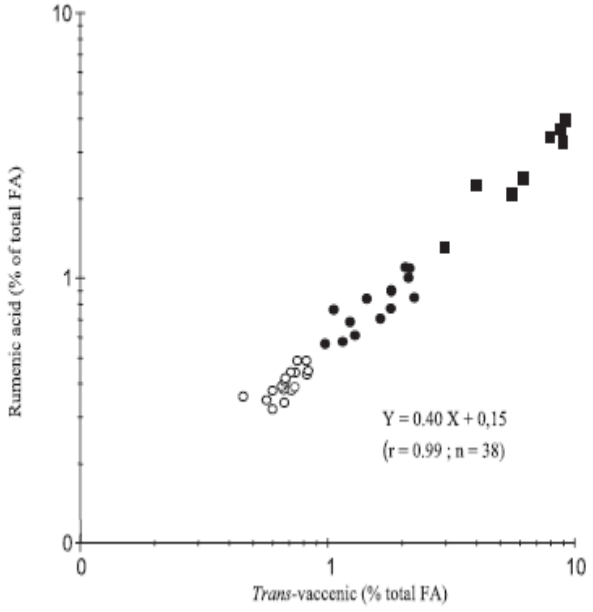


Table IV. Interactic **Table 2.3:** ge nature and vegetable oil supplementation (5–6 % of diet DM) on goat milk yield and composition (adapted from [81])[1].

Forage	Maize silage			Alfalfa hay		
Oil	C[2]	LO	OSO	C[3]	LO	OSO
Milk yield ($kg \cdot d^{-1}$)	3.62	3.95	3.61	3.65	3.61	3.54
Fat content ($g \cdot kg^{-1}$)	33.4^{b}	33.4^{b}	36.3^{bc}	29.7^{a}	36.9^{c}	35.1^{bc}
Fat yield ($g \cdot d^{-1}$)	121^{ab}	129^{a}	132^{a}	108^{b}	134^{a}	123^{ab}
Fatty acids (w% of total FA)						
C4:0	2.2^{ab}	2.8^{d}	2.6^{cd}	2.2^{ab}	2.4^{bc}	2.2^{ab}
C6:0	2.5^{bc}	2.6^{c}	2.4^{bc}	2.3^{b}	2.1^{ab}	2.1^{ab}
C8:0	2.8^{d}	2.6^{cd}	2.4^{bcd}	2.4^{b}	2.0^{a}	2.0^{ab}
C10:0	10.0^{c}	8.1^{ab}	7.4^{a}	8.8^{b}	6.1^{a}	6.5^{a}
C12:0	4.7^{b}	3.0^{a}	3.0^{a}	4.6^{b}	2.7^{a}	2.9^{a}
C14:0	11.7^{c}	8.2^{b}	8.4^{b}	12.2^{c}	7.6^{a}	8.4^{b}
C16:0	28.8^{b}	18.5^{a}	18.7^{a}	31.1^{c}	18.2^{a}	17.8^{a}
C18:0	7.5^{b}	9.5^{c}	13.8^{b}	6.0^{a}	10.8^{d}	12.7^{e}
C18:1 t10	0.24^{a}	2.97^{c}	2.20^{c}	0.07^{a}	0.29^{a}	0.70^{b}
C18:1 t11	1.18^{ab}	6.18^{c}	1.88^{ab}	0.45^{a}	8.80^{d}	2.12^{b}
C18:1 c9	15.7^{ab}	14.2^{a}	23.5^{c}	16.6^{b}	15.6^{ab}	27.7^{d}
C18:2 c9c12	2.0^{d}	1.5^{b}	1.4^{a}	2.3^{e}	1.7^{c}	1.6^{b}
C18:3 c9c12c15	0.32^{b}	0.69^{d}	0.17^{a}	0.60^{d}	1.37^{e}	0.42^{c}
C18:2 c9 t11	0.59^{ab}	2.42^{c}	0.80^{ab}	0.33^{a}	3.22^{d}	1.02^{b}
Desaturation index[4]	0.67^{c}	0.61^{ab}	0.63^{b}	0.73^{d}	0.59^{a}	0.68^{c}

[1] C, LO, OSO = control, linseed oil, oleic sunflower oil, respectively; twelve goats per group, except hay-control group ($n = 10$); results obtained after 5 weeks of lipid supplementation.
[2] Including 48% of concentrates.
[3] Including 44% of concentrates.
[4] 18:1c9:(C18:0 + C18:1c9); data in same row with similar superscript letters do not differ at $P < 0.05$ level.

2.1.10 Oils

Dhiman *et al*. (2000) showed that free oils (rich in linoleic or linolenic acid) in the diets of dairy cows increased the CLA content of milk. Free oil is not normally included in the diet as it produces inhibitory effects on microbial (particularly cellulolytic) activity in the rumen(Jenkins, 1993). In comparison, dietary oil in the form of intact seeds does not change milk CLA(Dhiman et al., 2000). Fish oils are more effective than vegetable oils, when equally added to the ration, at increasing the CLA concentration.

Milk fat depression commonly occurs when diets high in plant oils are fed (Davis and Brown, 1970)and high intakes of dietary fat may also cause milk protein concentration and yield to decrease. In this case the dietary fat adversely affects microbial fermentation and microbial protein yield thereby decreasing the supply of amino acids available for absorption by the cow(Palmquist and Jenkins, 1980).

Dhiman *et al*.(Dhiman et al., 1999a) showed also that feeding fish meal increased CLA content of milk by a small margin and Franklin *et al*.(Franklin et al., 1999) reported that cows fed marine algae had a greater concentration of CLA in their milk. In accordance with Davis & Brown (1970) with regard to plant oils, fish oil was toxic to rumen micro-organisms as it caused a decrease in concentration of milk fat. Furthermore, supplementation of fish oil at 200–400 g/d to dairy cows resulted in decreased DM intake (Doreau and Chilliard, 1997); this is likely to be a result of rumen microorganism toxicity.

As Chilliard and Ferlay (2004) reported, C18:2-rich vegetable oils (sunflower, soybean) highly increase milk rumenic acid content. This effect is linear as increasing amounts of soybean oil are added to the diet (up to at least 4% of diet DM) d Fig. 2.6). Adding rapeseed oil calcium salts to the ration increased also milk rumenic acid concentration. This confirmed that calcium salts of PUFAs are partially hydrogenated. Overall, vegetable oils increase milk rumenic acid more than extruded seeds, which in turn increase it more than raw seeds ((Chouinard et al., 2001), Tab.2.5). This effect is therefore more or less marked as PUFAs from free oil, extruded seeds or raw seeds disrupt rumen metabolism more or less intensively, consistently with the respective effects of oils or seeds on milk *trans*11-18:1 plus *cis*9,*trans*11-18:2 (Tab. 2.4; (Chouinard et al., 1997; Bayourthe et al., 2000).

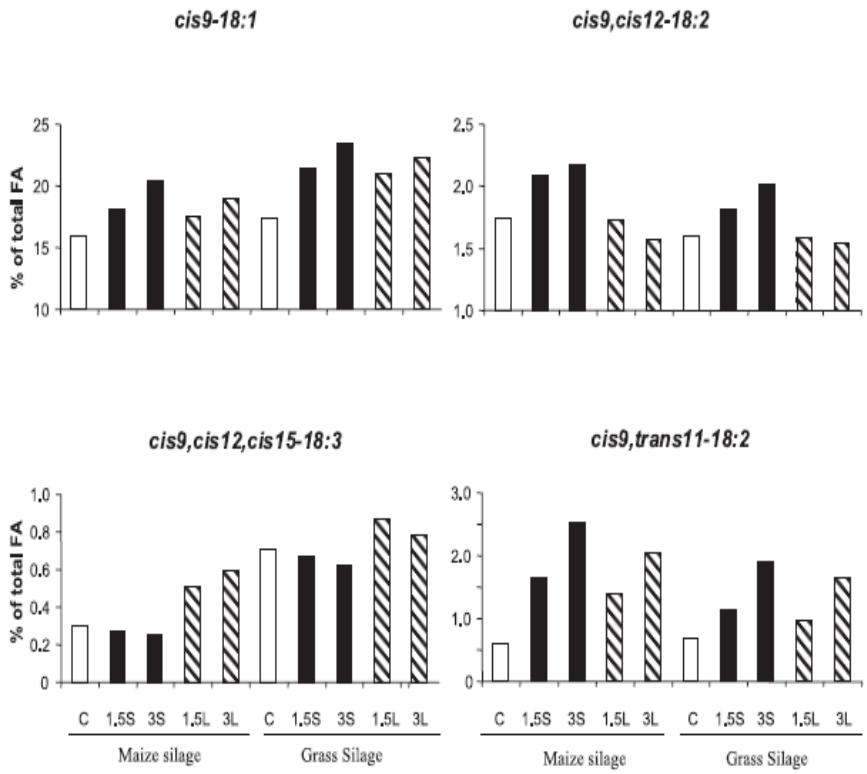


Figure 2.6. Effect of nature of forage, and nature dose of oil supplement on milk percentages of cis9-18:1, cis9, cis12, cis15-18:3 and cis9,trans11-18:2 in dairy cows (Ferlay A. and Chilliard Y., unpublished data).Abbrevations used: C, control (no oil);1.5S or 3S, diet supplemented with 1.5 or 4% of sunflower oil (18:2 rich); 1.5L or 3L, diet supplemented with 1.5 or 3% of linseed oil (18:3 rich)

Table II **Table 2.4:** lementation with oilseeds on milk yield, fat content and yield, and fatty acid composition in dairy cow[1].

Supplement	Linseed		Sunflower (18:2-rich)		Soybean			Rapeseed	
	Oil[2]	Seed[3]	Oil[2]	Seed[4]	Oil[5]	Raw[6]	Extruded[7]	Oil[8]	Seed[3]
Dietary lipids (%)	+3	+2.5	+3	+4.2	+2.3	+2.8	+1.8	+2.1	+2.3
Milk yield ($kg \cdot d^{-1}$)	+1.5	−1.7	+2.2	−2.5	+1.5	−0.3	−0.2	+0.8	−0.9
Milk fat content ($g \cdot kg^{-1}$)	−3.3	+0.3	−4.8	−7.1	−0.9	−2.1	+0.6	−2.8	−1.9
Milk fat yield ($g \cdot d^{-1}$)	−47	+30	−57	−259	+10	−40	+10	−70	−30
Milk fatty acids (% of total FA)									
C4:0	−0.1	nd[9]	−0.2	−1.2	+0.5	+0.3	+0.1	+0.4	nd
C6 to C8:0	−0.8	−0.6	−0.9	−2.1	−0.4	−0.2	−0.4	−1.1	−0.9
C10 to C14:0	−4.6	−3.2	−5.3	−8.6	−5.2	−3.7	−3.6	−2.7	−3.9
C16:0	−9.6	−3.2	−9.9	−8.7	−5.1	−7.5	−5.4	−10.7	−4.0
C18:0	+3.0	+3.8	+3.3	+4.1	+3.4	+0.8	+1.7	+4.0	+3.3
C18:1	+7.1	+4.0	+9.2	+15.8	+6.3	+7.9	+4.6	+13.6	+5.7
C18:1 c9	+4.0	nd	+5.2	(+9.7)[10]	nd	nd	+2.7	+10.6	nd
C18:1 t11	+3.1	−0.4	+4.0	(+7.4)[10]	nd	nd	+1.4	+3.0	+0.02
C18:2 c9 c12	−0.1	−0.7	+0.4	+1.3	−1.0	+0.9	+1.9	−0.1	−0.8
C18:3 c9 c12 c15	+0.2	+0.8	−0.1	+0.3	−0.04	−0.1	+0.3	+0.3	+0.02
C18:2 c9 t11	+1.2	−0.2	+1.6	nd	(+1.7)[11]	(−0.02)[11]	+0.5	+0.6	+0.01

[1] Difference between fat supplemented and control groups.
[2] A. Ferlay and Y. Chilliard, unpublished data, 47.5 or 59.8% silage, 12.9 or 4.7% grass hay, 36.7 or 32.4% concentrate, and 3% of oil for diets based on grass or maize silage, respectively.
[3] [91] 43.9% alfalfa silage, 11.8% barley silage, 8.3% ground linseeds (3.3% oil) or rapeseeds (3.4% oil), and 36.0% concentrate.
[4] [92] 40% maize silage, 15% alfalfa hay, 19% concentrate, and 21% of rolled sunflower seeds (9.8% oil).
[5] [93] 30% alfalfa silage, 20% maize silage, 47.7% concentrate, and 2.3% oil.
[6] [94] 42% dehydrated alfalfa pellets and long alfalfa hay, 16% maize silage, 14.7% ground raw soybeans (2.9% oil), 27.3% concentrate.
[7] [95] 25% alfalfa silage, 25% maize silage, 39.4% concentrate, and 10.6% extruded soybeans (2.1% oil).
[8] [96] 17.5% alfalfa haylage, 30.7% maize silage, 48.5% concentrate, and 3.3% oil.
[9] Nd, not determined.
[10] All *cis*-18:1 or *trans*-18:1 isomers.
[11] [97] 34% alfalfa silage, 17% maize silage, 45.4 or 31.0% concentrate, and 3.6% soybean oil or 18% raw cracked soybeans (3.6% oil), for diets supplemented with oil or soybeans.

Table III. Milk yiel **Table 2.5:** on in goats fed a low forage diet[1], supplemented or not with oils or whole crude oilseeds during 11 weeks[2] (7 goats per group) (adapted from [7]).

Diet	Control	Linseed oil	Linseeds	Sunflower oil	Sunflower seeds	Lupine seeds	Soybeans
Milk yield[3] ($kg \cdot d^{-1}$)	2.86	3.12	2.91	3.15	3.11	3.16	3.37
Fat content ($g \cdot kg^{-1}$)	25.5^{a}	28.6^{b}	31.5^{b}	30.7^{b}	31.3^{b}	29.2^{b}	29.6^{b}
Fat yield ($g \cdot d^{-1}$)	72^{a}	90^{b}	93^{b}	94^{b}	95^{b}	92^{b}	97^{b}
Fatty acids (w% of total FA)							
C4 + C6 + C8	7.9^{b}	7.5^{ab}	7.8^{b}	7.7^{b}	6.9^{a}	7.4^{ab}	7.1^{ab}
C10 + C12 +C14	24.9^{b}	16.3^{a}	18.0^{a}	17.3^{a}	16.9^{a}	18.6^{a}	16.9^{a}
C16:0	25.8^{c}	16.9^{a}	19.0^{b}	18.2^{ab}	18.7^{b}	19.4^{b}	19.6^{b}
C18:0	9.0^{a}	13.8^{b}	15.2^{bc}	13.0^{b}	15.9^{c}	13.5^{b}	16.6^{c}
C18:1 t11	0.95^{a}	2.92^{bc}	1.28^{a}	3.94^{c}	2.30^{b}	0.66^{a}	0.85^{a}
C18:1 c9	19.1^{a}	22.6^{ab}	24.7^{bcd}	20.8^{a}	23.9^{bc}	27.4^{d}	26.3^{cd}
C18:2 c9 c12	2.2^{c}	2.2^{bc}	1.9^{b}	3.4^{e}	3.0^{d}	1.6^{a}	3.3^{de}
C18:3 c9 c12 c15	0.41^{a}	1.68^{d}	1.24^{c}	0.49^{a}	0.51^{b}	0.63^{b}	0.40^{a}
C18:2 c9 t11	0.56^{b}	1.38^{d}	0.60^{b}	2.28^{e}	0.84^{c}	0.28^{a}	0.40^{ab}
AI[4]	2.92^{c}	1.21^{a}	1.61^{b}	1.36^{a}	1.48^{ab}	1.72^{b}	1.52^{ab}
Desaturation index[5]	0.69^{c}	0.63^{b}	0.62^{ab}	0.61^{ab}	0.59^{a}	0.67^{c}	0.61^{ab}

[1] Natural grassland hay (30%) and concentrates with or without oils or oilseeds (70%).
[2] 3.4 ± 0.6% added lipid in DM intake (supplemented-control).
[3] Data in same row with similar superscript letters do not differ at $P < 0.05$ level.
[4] Atherogenecity index, (C12 + 4 C14 + C16):(sum of unsaturated FA).
[5] C18:1c9:(C18:0 + C18:1c9).

CLA proportions increased from 0.2–0.6% with the control diet to 1.5–2.7% with diets supplemented with fish oil (200–300 g·d–1, (Chilliard et al., 2000; Chilliard et al., 2001). It is likely that the PUFAs (EPA and DHA) of those oils increase *trans*11- 18:1 concentration in the rumen, probably through inhibiting the reduction of that FA into stearic acid. That would explain why the combination of vegetable oils and fish oil strongly increased milk CLA content (Palmquist, 2001; Whitlock et al., 2002). Rumenic acid secretion in milk is correlated to the duodenal flow of *trans*11-18:1 (Loor et al., 2004). Furthermore, there is a strong linear correlation between milk rumenic acid and *trans* 11-18:1 concentrations under a wide variety of diets, either in goats or cows. However, the milk rumenic acid/*trans*11-18:1 ratio could be decreased with fish oil supplementation (Chilliard et al., 2001). In this case, the very high concentration of *trans*11-18:1 may exceed the desaturation capacity of the mammary gland, or fish-oil specific FAs(EPA, DHA or intermediate hydrogenation compounds) may inhibit Δ-9 desaturase activity. Previous data (Bauman et al., 2000a) suggested that the milk rumenic acid response to lipid supplementation could be transient, with a maximum during the second week after the beginning of lipid supplementation. This was recently confirmed (Ferlay et al., 2003a) and observed that the rumenic acid response to lipid supplementation was higher with hay diet than maize silage diet, and that the decrease after 3 weeks of supplementation was accompanied by an increase in milk fat *trans*10-18:1 percentage, that was more marked with maize silage (Fig. 6). This confirms the theory proposed by Bauman and Griinari (Bauman and Griinari, 2000) and Bauman et al (Bauman et al., 2001) on the *trans*10-18:1 pathway which would decrease the yield of ruminal vaccenic acid and its availability for rumenic acid synthesis in the mammary gland. This rises also the question of the sustainability of high CLA responses in dairy cattle, and further studies are needed on interactions between dietary fiber, starch, fatty acids and other components. No data are available on the short-term kinetics of CLA response in goat milk. However the high CLA levels obtained after 5 weeks of lipid supplementation have been confirmed in the same goats after 9–10 weeks of supplementation This shows that goat species is a very good responder and that its milk rumenic acid response is stable during at least 2–3 months. Indeed, goat milk rumenic acid varies largely according to feeding factors. Few data are available on the influence of feeding on the various milk CLA isomers. Rumenic acid (*cis*9,*trans*11-CLA) is classically the one whose concentration is the most variable because of the importance of its mammary synthesis by Δ-9 desaturase. In addition, this enzyme synthesizes *trans*7,*cis*9-CLA, quantitatively the second isomer present in milk. That isomer is increased in cow by low-fiber diets supplemented with soybean oil (Piperova et al., 2000) and probably in goats by high-oleic sunflower oil supplementation (Ferlay et al., 2003b). Low-fiber diets increase *cis*11,*trans*13- and *cis*9,cis11-CLA isomers, whereas linseed oil increases *cis*9,*cis*11-, *trans*11,*cis*13- and *trans*11,*trans*13-CLA, as well as *trans*13-18:1, *cis*9,*trans*13-18:2 and *trans*11,*cis*15-18:2 (Loor et al., 2005). It should be stressed that the obtention of a high level of rumenic acid (3–4% of total FAs) is accompanied by high levels not only of vaccenic acid (8–10%) but also of other *trans*-isomers of C18:1 and conjugated or non-conjugated C18:2 (5–10%) and C18:3. The respective physiological roles of these various isomers and their possible nutritional interest to man have not been studied to date.

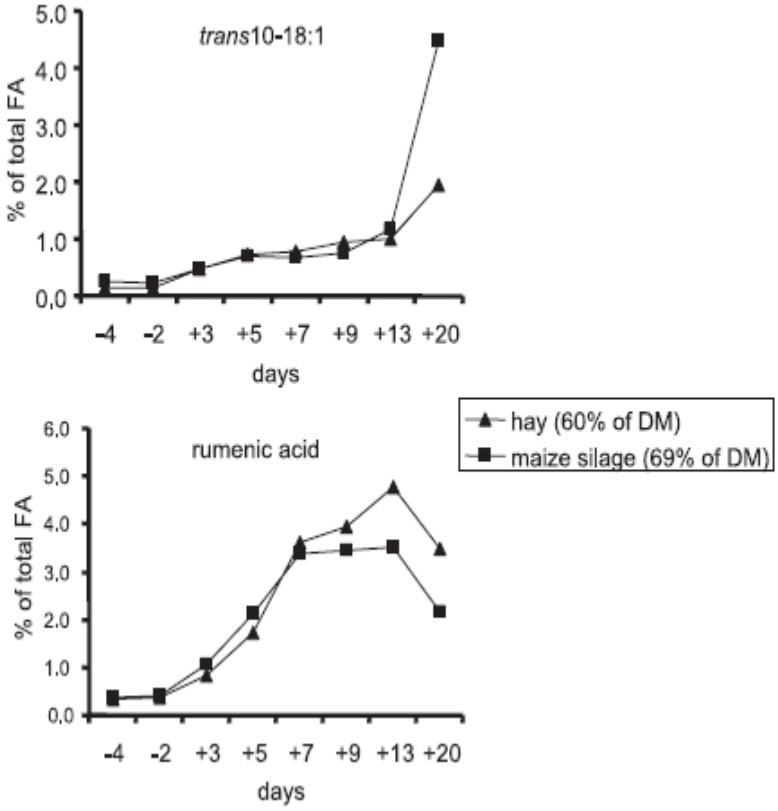


Figure 2.6. Effect of nature of forage on the kinetics of percentages of cow milk *trans*10-18:1 and
rumenic acid after the addition of oil into the diet (adapted from [80]).

2.1.11 Plant oils

Griinari *et al*.(Griinari et al., 1996)showed that the addition of dietary unsaturated fatty acids such as maize oil and changing forage:concentrate ratios enhanced the CLA content of milk fat. In contrast, Dhiman *et al*. (Dhiman et al., 1999a)found that supplying an additional 10 g CLA/kg fat in the diet through high-oil maize and high-oil maize silage did not influence the CLA content of milk.

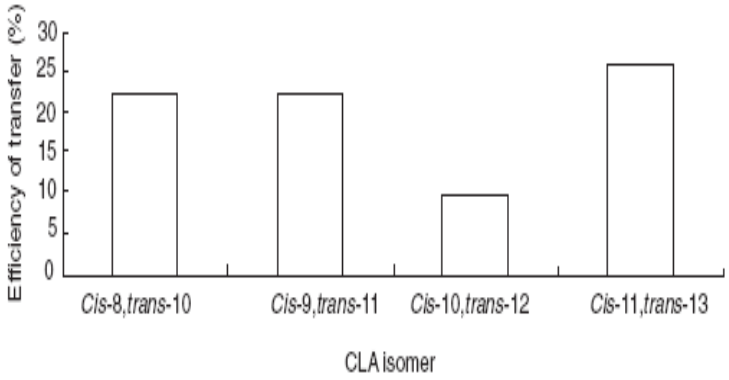
Stanton *et al*.(Stanton et al., 1997) reported that a supplement of full-fat rapeseed (high in oleic acid) caused a greater increase in CLA content of milk than did soybean oil (high in linoleic acid). Stanton *et al*. (1997) found that rapeseed supplementation resulted in an increase of 650 g CLA/kg milk over non-supplemented control, but the total fat concentration was not reported. It is not known whether the effect of rapeseed oil was due to its relatively low linoleic acid content or an effect of large amounts of oleic acid. Feeding vegetable oils or seeds increased milk fat content in goats (Chilliard and Bocquier, 1993) whereas it generally decreased it in cows (Chilliard, 1993).

These differences observed with goats could be related to differences in the metabolism of *trans* fatty acids in the rumen or in the mammary gland..

Kelly *et al*.(Kelly et al., 1998a) reported that feeding sunflower oil (high in linoleic acid) increased CLA concentrations to 24·4 g/kg milk fat compared with values of 13·3 and 16·7 g/kg fat for high-oleic (peanut oil) and high- inolenic acid oils (linseed oil), respectively. These studies suggested that, given an adequate dietary intake of linoleic acid, dietary constituents that provide rumen substrates for the optimal growth of bacteria producing linoleic acid isomerase would maximise CLA output. Feeding linseed oil (linolenic-rich) greatly increased CLA content in milk fat (Chouinard et al., 1998; Dhiman et al., 2000) and was showed to be as efficient as sources of linoleic acid (Chilliard et al., 2000). Linolenic acid is not a precursor of CLA in the rumen and it has been suggested (Chilliard et al., 2000) that feeding linseed oil results in a large increase in the production of rumen *trans*-11-18:1, which can be used by the mammary gland for CLA synthesis (Fig. 2.7).

When soybean oil was offered twenty-four times daily instead of twice, the milk fat content increased and the percentage of *trans*-18:1 decreased, whereas that of 18:0 increased (Banks et al., 1980). This suggests that rumen hydrogenation was more complete and that the milk CLA synthesis was probably decreased. The CLA content of milk and cheese may also be increased by the addition of extracted soybeans and cottonseed to the diets of dairy cows. Dhiman *et al*. (Dhiman et al., 1999a)suggested that to make oil more readily available for digestion, the soybeans and cottonseeds can be processed through an extruder to rupture the seeds. Dhiman *et al*.(Dhiman et al., 1999b) reported that contents of CLA in milk and cheese were doubled from 0.34g/100 g fatty acid to 0.69 g/100 g fatty acid by the inclusion of full-fat extruded soybeans. Full-fat rapeseed supplementation of diets fed to lambs or dairy cows have been showed to increase CLA content of meat (Mir et al., 2000) and milk(Stanton et al., 1997) respectively.

Fig. 2.7. Apparent efficiency of abomasally-infused conjugated linoleic acid (CLA) isomers into milk fat of dairy cows. Adapted from Chilliard *et al*. (2000).



2.1.12 Grazing

It is well known that, compared with non-grazing cows, grazing produce milk with higher proportion of CLA, vaccenic (VA) and linoleic acids (Dewhurst et al., 2006). This invests fresh forage with added value and highlights grazing as worthwhile natural production system. Nevertheless, the milk CLA proportions measured in cows at pasture are variable (0.5 to 1.7%)(Chilliard and Ferlay, 2004). Milk CLA concentration increases with green grass availability (Stanton et al., 1997; Dhiman et al., 1999a) and is further increased by lipid supplements (Schroeder et al., 2004). Young grass high C18:3 concentration and low fiber content probably combine to increase CLA and *trans*-18:1 production. Also, the particular botanical composition of natural highland meadows seems to promote high milk CLA concentrations (up to 2.4%,(Boylston T.D, Lin H., Luedecke L.O., Shultz T. D., 1996), whereas a botanical composition effect of cultivated swards appears to be low. Comparisons in cows suggest that the milk rumenic acid response to lipid supplementation differs between forages, with hay >maize silage > grass silage. Further studies are needed to confirm and explain these interactions.

Morales- Almaráz et al.(Morales-Almaráz et al., 2010) reported that the pasture intake reduced the proportion of SFA and increased that of UFA (mainly MUFA) in the milk. This might be explained by the higher intake of C18:2 and C18:3 in fresh forage and their biohydrogenation in the rumen. Bargo et al. (Bargo et al., 2006) reported high intakes of C18:3 and C18:2 in cows feeding on pasture. Also Bauman et al. (Bauman et al., 2000b)showed that ruminal lipolysis and free FA biohydrogenation led to drastic reductions (70-90%) in dietary PUFA via their transformation into trans-isomers of MUFA (especially VA) or SFA (mainly stearic acid). Morales- Almaráz et al. showed that the use of the grass does not reduce milk yield, and fat is healthier also, which could lead to an increase in value added of milk produced.

Seasonal variations in the CLA content of milk are very marked, with values during the summer often up to two or three times higher than during the winter(Jahreis et al., 1997; Parodi, 1999). From studies of other *trans* fatty acids a seasonal effect for example on *trans* vaccenic acid content in milk fat is known (Precht et al. 1995). Moreover, Jahreis *et al*. (Jahreis et al., 1997) reported that grazing of fresh polyunsaturated fatty acid-rich grass increased the formation of both CLA and *trans*-vaccenic acid in milk.

The high content of CLA in milk from cows offered pasture has also been attributed to the linoleic acid content of the forage although the proportion of linoleic acid is low compared with α-linolenic acid (Garton, G.A. 1960). Feeding sheep diets high in α-linolenic acid increases the rumen content of *trans*-18:1 fatty acid as a result of incomplete hydrogenation (Czerkawski et al., 1975).

. Jahreis *et al*. (Jahreis et al., 1997) reported that milk from cows grazing pasture had higher CLA content compared with cows offered maize silage and high-cereal-based concentrates. Increasing the proportion of grazed grass from pasture in the diet of dairy cows linearly increased the CLA content of milk (Dhiman et al., 1999).

Cows grazing pasture permanently had five times more CLA compared with cows fed total mixed ration containing conserved forage–grain (50:50, w/w). Conversely, feeding hay did not influence milk CLA content. Boylston *et al*. (1996) did not observe any seasonal CLA variations in dairy cows fed the same total mixed ration throughout the year. Therefore, it seems seasonal variation in CLA content of milk can be attributed to the proportion of grazed grass in the diet. Indeed, Precht &

Molkentin (Precht et al. 1997) found that milk fat from cows consuming pasture contained a mean of 12,0 g CLA/kg milk compared with 4,5 g CLA/kg in milk from cows fed hay, silage and concentrates. Jahreis *et al*. (Jahreis et al., 1997) suggested that the reason that the CLA content in the milk from cows living indoors all year was low compared with pasture fed cows, was that the diet fed to these cows was low in polyunsaturated fatty acids, and therefore a deficiency of substrate for biohydrogenation by rumen bacteria existed. Although not the only determinant, the amount of dietary polyunsaturated fatty acid determines the generation of *trans* fatty acids by rumen bacteria.

Milk from grazing dairy cows had a higher content of polyunsaturated fatty acids (PUFA), especially more conjugated linoleic acid (CLA), and lower proportions of saturated fatty acids (SFA) than milk from silage-fed cows. Nevertheless, few researches are made trying to evaluate the influence of grazing time in milk fatty acids (FA) composition and its variation across the grazing season. Roca-Fernández, A et al. (Roca-Fernandes et al.,2010) investigated the effect of different proportions of grazing in the ration of dairy cows and its variation across the season on milk FA profile and found that grazing swards 24-h (G24) caused a decrease ($P < 0.05$) in short and medium chain fatty acids (SCFA, 8.34 and MCFA, 39.24 g 100 g−1 of FA in milk, respectively) and an increase ($P < 0.05$) in long chain fatty acids (LCFA, 42.29 g 100 g−1 of FA in milk). The highest ($P< 0.05$) content of monounsaturated and polyunsaturated fatty acids in milk (MUFA, 25.20 and PUFA, 4.24 g 100 g−1 of FA, respectively) were observed in G24. The CLA content showed an increase ($P < 0.05$) with grazing time, ranging from 0.72 to 1.23 g 100 g−1 of FA in milk for Grazing 12h.

2.1.13 Forage: concentrate ratio

Lower forage: concentrate ratios in dairy cattle diets have also been showed to increase CLA concentration in milk (Jiang et al., 1996) . Jiang *et al*. (1996) were able to double the CLA content from 5·04 to 11·28 g/kg fat by feeding a higher concentrate: roughage ratio to dairy cows, without appreciably increasing the percentage milk fat.

2.1.14 Animal factors

Depending on feeding and milk performance, genetic constitution and stage of lactation, the composition of bovine milk fat is subject to strong variations. Indeed, Doyle (Doyle et al., 1998) reported that survey results demonstrate that CLA concentration in milk from New York, USA, herds ranges from 2·2 to 20·1 g/kg fat. Moreover, Lawless *et al*. (Lawless et al., 1999) proposed that the influence of cow breed on milk CLA is either not significant or limited, with milk from Montbeliardes showing slightly higher values than Holstein–Friesians and Normandes offered grazed grass. Recently, Kelly *et al*. (Kelly et al., 1998) examined this variation for cows fed

either a pasture diet or a total mixed ration. Although they observed that individuals maintained relatively constant milk fat concentrations of CLA across time, there was a threefold variation in milk CLA content among individuals (2·4–7·0 g/kg milk fat) even though all cows were at a similar stage of lactation, consumed the same diet through a total mixed ration at similar intakes and produced similar amounts and composition of milk. The CLA content in milk fat from cows in the grazing group (Kelly et al., 1998) showed significantly ($P < 0·05$) higher individual cow variation as the proportion of pasture in the ration increased (3·0–9·0 and 6·3–18·1g/kg milk fat for total mixed ration and grazing respectively). Kelly *et al*. (Kelly et al., 1998) reported substantial individual variation (9·9–51·7 g CLA/kg fat) in cows at the same stage of lactation that consumed a total mixed ration supplemented with 53 g sunflower oil/kg DM. The variation was significantly lower for supplements of peanut oil and linseed oil. The results suggest additional factors such as individual genetic regulation of rumen microflora may operate (Moore et al., 1993).

Stanton *et al*. (Stanton et al., 1997) reported that certain breeds of cattle and some individual cows appear to be more efficient at incorporating CLA into milk, with a range of 3–25 g/kg milk fat, when offered grazed grass. Older cows (>4 years old) tended to produce milk with more CLA, as did those fed a higher grass allowance.

2.1.15 Effects of feeding factors on the sensory quality of dairy products

Before recommending dietary modifications to breeders to modify milk FA composition, it has to be ascertained that such practices would not be detrimental to the sensory quality of dairy products. Cow milk sensory analysis was performed by two comparisons (triangular tasting tests) of crude, unskimmed milk from groups of cows receiving different diets. Differences exist between milk produced by grazing cows and others obtained from cows fed hay-based or high-concentrate diets. Also, milk samples from cows fed grass silage were identified as different from milk derived from hay-based or maize silage diets. Supplementing maize silage-based diets with 3% sunflower oil led to moderate but noticeable sensory differences, whereas the distinction is no longer possible with grass silage-based diets. Addition of 5% linseed oil to a maize silage-based diet was easily identified. Adding 2.5% fish oil induced sensory defects.

Cheese or butter sensory qualities are defined by their aspect and texture, in addition to their organoleptic properties (flavour). Certain FAs exert a specific effect on the hardness and spreadability of butter. Increased palmitic acid concentration combined with a decrease in short-chain FAs

lead to lower spreadability. The 16:0/ *cis*9- 18:1 ratio is the most accurate indicator of butter firmess. So, pasture grazing led to more spreadable butter with a reduced 16:0/*cis*9-18:1 ratio (Hurtaud et al., 2002). Also, butter derived from hay-based diets was rated as less firm and with less flavour than butter produced from a maize silage-based diet, in paralled with an increase in mono- and poly-unsaturated FAs with the hay-based diet. With regard to cheese, the type of pasture induces a modification of milk fatty acid composition, which affects cheese texture. Unsaturated fatty acid-rich milk produces less firm Abondance cheeses. Cantaltype cheeses produced with milk derived from high-concentrate diets are firmer than those obtained from natural grass, probably because of their higher C18:0 and lower PUFA

concentations. Pasture led to more “animal” and less “bitter” and less “sour” odour, such differences being less marked with pasteurized milk (Verdier-Metz et al., 2002). Hay, compared with maize silage, led to Emmental cheeses containing more mono- and poly-unsaturated FAs but less firm and piquant in taste, with a less pronounced “bone-fruit” flavour. Other experiments have also showed the effects of forage and lipid supplements and their interactions on goat cheese flavour (Gaborit et al., 2002). Linseed oil or unflower oil supplementation (5–6% of the ration) reduces the “goaty” taste in milk or fresh cheese, linked to the lower secretion of lipase and reduced post-milking lipolysis(Chilliard et al., 2003). Also, more bitter, piquant, oxidized or fishy flavours may occur, especially with the hay + linseed oil combination hich maximizes milk C18:3 concentration .

2.1.16 Conclusion

Feeding factors make it possible to vary milk FA composition in many ways. Recent advances in the knowledge of FA synthesis mechanisms (digestion and metabolism) and their putative physiological effects in human consumers have significantly boosted ongoing research and potential applications. As regards ruminant nutrition, the aim is to better understand the effects of using grass-based diets, new combinations of feedstuffs in concentrates, and oil seed technology and processing. However, very few direct comparisons have been made between the main types of basal diets (different types of forages, starchy concentrates, etc.) combined with various lipid supplements (oils, seeds, technological processing and lipid dose added to the basal diet). So, the trends need to be confirmed and specified. However, it is clear that the plasticity of milk fat composition is very large, according to numerous interactions between forage-concentratesoils- minerals-vitamins, time after dietary changes, as well as ruminant species (Chilliard et al., 2003), on almost all major and minor FAs, including several *trans* isomers of C18:1, C18:2 and C18:3. Insofar as human nutritional recommendations may still vary in the coming years, and as the putative effect of a large number of specific FAs (e.g. *trans* isomers of C18:1, C18:2, C18:3) on human health are not yet known, animal nutritionists have to keep exploring the response patterns of major and minor milk fatty acids and to model their synthesis mechanisms. At the same time, the side effects of the various dietary practices on health safety (presence of antinutritional factors, variations of nutrients with pro-oxidant effects, etc.), on technological and sensory quality as well as on the image of dairy products need to be better assessed.

2.2 Effect of negative energy balance and liver metabolism on reproduction

The onset of lactation in dairy cows is characterized by a marked increase in energy demands for milk synthesis, which along with the gradual decrease in dry matter intake (DMI) that occurs over the last three week before calving, results in negative energy balance at parturition. This negative energy balance generally persists through the first 6 to 8 weeks postpartum, and often is energetically equal to one-third or more of the milk energy secretion (Bauman and Bruce Currie, 1980). In order to meet the glucose requirements for milk synthesis in early lactation, liver rates of gluconeogenesis are up regulated and circulating insulin decreases, thus resulting in lower glucose utilization by peripheral tissues and greater availability for the mammary gland. The cow in negative energy balance has lower circulating concentration of glucose, insulin and insulin-like growth factor I (IGF-I), and high concentration of growth hormone (GH) that support milk synthesis (Grummer, 1995; Bauman and Griinari, 2000).

Endocrine changes at parturition along with the marked decrease in DMI results in an extensive mobilization of body fat reserves and an increase in circulating concentration of non-esterified fatty acids (NEFA). Plasma NEFA concentration increases about two-fold from day 17 to day 2 prepartum, then rise dramatically until completion of parturition, and decrease rapidly afterwards, but concentrations remaining higher than before calving (Grummer, 1995). The liver takes up circulating NEFA in proportion to their concentration in the blood, and they can be completely oxidized to CO2, converted to ketone bodies for use in peripheral tissues, esterified and exported as very low density lipoprotein (VLDL), or accumulate in hepatic tissue as triglycerides

(Bell, 1995; Goff and Horst, 1997; Drackley, 1999). Carnitine palmitolytransferase 1 (CPT1) constitutes an important regulatory step in the oxidation, and its activity is increased postpartum (Aiello et al., 1984). An alternate pathway for hepatic oxidation of NEFA is the peroxisomal β-oxidation which differs from mitochondrial oxidation in the production of hydrogen peroxide rather than NADH, with a net effect that less energy is captured and more heat is released (Drackley, 1999).

Esterification of NEFA occurs in the smooth endoplasmic reticulum, where triglycerides and phospholipids are attached to Apo B forming nascent VLDL. These are carried to the Golgi apparatus for terminal glycosylation, and finally travel to the cell surface where they are released into the blood via the space of Disse to transport lipids to the peripheral organs (Bauchart, 1993). The ability of the liver to secrete triglycerides as VLDL is proportional to its lipogenic capacity, which is very limited in cows. (Pullen et al., 1990). When the rate of esterification exceeds the rate of triglyceride export VLDL, accumulation of triglycerides occur causing development of fatty liver syndrome (Grummer,1993; Goff and Horst,1997). Cows that develop fatty liver have decreasead activity of gluconeogenic enzymes leading to reduced blood glucose concentration (Murondoti et al., 2004). Decreased gluconeogenic capacity may be consequence of higher DMI prepartum often observed in cows that develop fatty liver; high availability of starch can result in less adaptation in rates of gluconeogenesis (Rukkwamsuk et al., 1999).

2.2.1 Effect of negative energy balance on reproductive function

During negative energy balance in early lactation homeorhetic controls favor milk production over reproduction. It has been established that negative energy balance during the first 20d of lactation is inversely related to days to first postpartum ovulation (Butler et al., 1981) and that changes in energy balance over time trigger the first postpartum ovulation (Canfiled and Butler, 1990). Early resumption of cycling after parturition is advantageous because longer intervals from first ovulation to breeding allow for more optimal restoration of uterine environment increasing pregnancy rates (reviewed by Thatcher et al., 2006).

After parturition, the decrease of estrogen restores follicle-stimulating hormone (FSH) concentrations; this sequence is responsible for the development of a new follicular wave and occurs regardless of energy status (review of Beam and Butler, 1999). Most dairy cows appear to develop dominant follicle during the second week postpartum, and the fate of the first dominant follicle will determine interval to first ovulation. The dominant follicle can have three outcomes: ovulation, regression followed by a new follicular wave, or failure to ovulate with the dominant follicle becoming cystic. The first ovulation postpartum is dependent on resumption of luteinizing hormone (LH) pulse, and re is affected by negative energy balance. LH frequency is reduced in those cows that develop anovulatoryfollicles in the first follicular wave postpartum, but energy restriction does not alter pituitary GnR>h receptor density; rather, evidence suggests a hypothalamic locus for the effect of decreased energy intake (review of Beam and Butler, 1999).

During early lactation, GH levels are high, but expression of GH receptors in the liver is decreased resulting in low levels of IGF-I. This is an indicator of nutritional status to hypothalamic-pituitary-ovarian axis (reviewed by Beam and Butler, 1999). The low expression of GH receptors in the liver, during early lactation is restored by insulin (review of Butler et al., 2003) and it has been reported that diets that result in high concentrations of circulating insulin reduce the intervals to first postpartum ovulation when compared with isocaloric diets that had lower levels of insulin (Gong et al., 2002). LH pulsatility ensures sufficient estrogen production and thus feeds back to the brain to induce LH surge. Cows with poor body condition score (BCS) have decreased diameter of dominant follicle and low LH pulse frequency (reviewed by Roche, 2006). Low intrafollicular concentrations of IGF-I result in a reduction in LH receptors in the granulosa cells and decreased estradiol synthesis by the follicle (review of Beam and Butler, 199).

Besides resumption of ovulation, energy status also influence early embryo survival by affecting the quality of the ovarian follicle. NEFA impact negatively the proliferation and maturation of granulosa cells and embryonic development in vitro (Jorritsma et al., 2004), and intrafollicular concentrations of urea, triglycerides, glucose, insulin, β-hydroxybutyrate (BHB) and NEFA are correlated with serum concentrations (Leroy et al.,2004). Development of a small follicle to ovulatory stage take about 40 to 50 days; therefore, extreme changes in circulating metabolites during early lactation may affect the follicular environment resulting in the mature follicle having an impaired fertility. Detrimental effects on stereidogenesis of the dominant follicle have been reported as a consequence of heat stress during follicular development, suggesting that viability of the mature follicle is affected by the conditions during follicular development (Roth et al., 2001).

High progesterone levels are important during the early luteal phase and result in increased embryo development and survival (> Mann et al., 2006). Progesterone is metabolized in the liver and excreted in feces, urine and milk. It has been reported that plasma progesterone concentrations decrease after feed consumption due to an increase in metabolic rate and blood flow to the liver, and this effect was eliminated if multiple small meal were fed.(Vasconcelos et al., 2003). In high producing cows with high DMI, rate of progesterone metabolism may be greater. Progesterone profile can also be affected by negative energy balance. Pushpakumura et al. (2003) reported that cows with low circulating lower IGF-I than cows with normal progesterone; in addition, cows that did not get pregnant in this study had higher BHB during the pre-breeding season and a greater BCS loss during the first 7 weeks postpartum. Metabolic disorders during early lactation can also predispose the cows to reproductive problems. For example, high concentration of ketones are associated with reduced uterine contraciont resulting in retained placenta and endometritis (revied by Roche, 2006) and elevated liver triglycerides (> 50mg/g) is related to longer periods to first estrus and conception (Jorritsma et al., 2000).

2.2.2 Effect of fat supplementation in reproduction

In order to reduce the extent of the negative energy balance during early lactation, nutritional approaches have focused on increasing energy density of the diet to overcome the decrease in dry matter intake. This can be achieved through partial substitution of forages with more energy dense concentrates and fat supplements (Palmquist and Conrad, 1978; Schingoethe and Casper, 1981). However, greater amounts of concentrate often predispose transition cows to displaced abomasums acidosis and laminitis (Ostergaard and Sorensen, 1998; Owens et al., 1998). Therefore, the addition of fat supplements (about 3% of DMI) in early lactation has become a standard practice to increase energy density of the diet. Improvements in reproductive variables are not consistently observed across experiments, partly because a slight decrease in DMI often occurs when fat supplements are included in the diet. Therefore, an increase in the dietary energy density does not always result in an improvement in the net energy balance. In addition, the specific fatty acids present in the fat supplements can impact ovarian and uterine function. The supplements more commonly used include whole oilseeds, yellow grease, fish oil, prilled fat and CA salts of fatty acids. These supplements differ widely in their fatty acid profile, and also in the percent of unsaturated fatty acids that escape rumen biohydrogenation and are subsequently absorbed by the cow. (Staples, 1998; Butler et al., 2005).

2.2.3 Effect of fatty acids in prostaglandin synthesis

Linoleic (C18:2 n-6) and linolenic (C 18:3 n-3) acids are essential faty acids that have important biological functions and must be provided by the diet. Linoleic acid is the precursor of arachidonic acid while linolenic acid is precursor of eicosapentaenoic acid (EPA). The elongation and desaturation of PUFA are mediated by the action of three enxymes: Δ-6 desaturase, elongase and Δ-5 seaturase (reviewed by Cunnane, 2003).

Prostaglandins are biologically active compounds synthesized from 20-carbon fatty acids, and they have a variety of physiologica functions.

Prostaglandin E2 (PGE2) has and inhibitory effect in ovulation while prostaglandin F2α (PGF2α) has a luteolytic action (reviewed by Abayasekara and Wathes,1999). Maternal recognition of pregnancy in the bovine is dependent on the secretion of interferon tau (IFNτ) by the developing embryo. IFNτ inhibits the secretion of PGF2α by the uterus preventing luteolysis. However, insufficient communication between the conceptus and maternal tissue can result in increased PGF2α synthesis, luteolysis and embryonic loss (reviewed by Wolf et al.; 2003). It has been calculated that 32% of total embryo losses occur at this stage of pregnancy (Dunne et al., 2000).Therefore, modulation of prostaglandins by dietary fatty acids may improve embryo survival.

Synthesis of prostaglandins involves the release of 20-carbon PUFA from the phospholipids in the cell membranes by the action of phosopholipase A2,phospholipase C and diglyceride lipase.These PUFA are then converted to prostaglandins by the action of prostaglandin synthase also called cycloxigenase (COX). Arachidonic acid (C20:4 n-6) is the precursor for the 2-series of prostaglandin while EPA (C20:5 n-3) is used for the synthesis of the less active 3-series prostaglandis. Therefore, dietary PUFA may alter uterine prostaglandin synthesis through substitution of arachidonic acid in the phospholipid fraction, competition for the action of key enzymes and /or direct inhibition of COX2. Reduction in total PGF2α production or replacement by the less bioactive PGF3α amplifies the inhibition by the early embyo helping to prevent luteolysis (reviewed by Abayasekara and Whates, 199; Mattos et al.2000).

An approach commonly used to evaluate the effect of PUFA on uterine prostaglandin synthesis is to measure plasma concentration of 13,14 dihydro,15-keto PGF2α (PGFM), the metabolite of PGF2α, at frequent intervals before and after an intravenous injection of ocytocin. Decreased PGFM response to this type of oxytocin challenge was reported by Oldick et al. (1997) in cows that were abomasally infused with yellow grease (high in linoleic and linolenic acid) for 35 days compared to cows that received an infusion of tallow (mainly saturated fatty acids). Similarly, Mattos et al (2002) observed a decreased PGFM response after 45 days of supplementation with a combination of menhaden fish meal and fish oil, which provided EPA and docosahexaeonic acid (DHA). However, these results were not consistent with the results obtained by Petit et al.,(20002), where cows supplemented with a 50:50 mixture of formaldehyde protected linseed and fish oil had higher circulating concentration of PGFM as compared to cows that received duodenal infusion of linseed oil, formaldehyde treated whole linseed, or calcium salts of fatty acids containing oleic and linoleic acids.

Effects of dietary supplementation of n-6 fatty acids on PGFM were evaluated by Robinson et al. (2002); they reported an increase in plasma concentrations of

PGFM after an oxytocin challenge when cows were supplemented with linoleic acid,but no differences when cows were supplemented with linolenic acid. In contrast, the same group reported that endometrial explants from cows fed diets containing increased response to an in vitro oxytocin challenge (Cheng et al., 2001). Incorporation of EPA and DHA in endometrial tissue has been observed after short periods of supplementation. For example, Burns et al., (20003) reported these fatty acids and Mattos et al. (2004) reported decreased plasmM 60h after parturition and incorporation of EPA and DHA in caruncular tissue when cows were fed a fish oil supplement from 21 days prepartum to 21 days postpartum. The extent of the reduction in prostaglandin production caused by dietary fatty acids can be influenced by exposure to progesterone, which is essential for PGF2α release in response to oxytocin; progesterone is thought to upregulated post-receptor signaling pathways or enzymes involved in prostaglandin sybthesis (Mann and Lamming, 2001). Wamsley et al. (2005) found that supplementation with fish meal decreased PGFM response to an oxitocin challenge only in cows with low luteal –phase progesterone concentration,whereas non change were observed when cows had high luteal-phase progesterone.

Recently, effects of other unsatured fatty acids on the reproduction of dairy cows have been explored. Conjugated linoleic acid (CLA) is a colletive term used to describe positional or geometric isomers of linoleic acid containing a conjugated fdouble bond system (Parodi, 1994) and they are formed as intermediateds during rumen biohydorgenation of linoleic acid. Two of the isomers that have been extensively studied are cis-9, trans-11 CLA, that has anticarcinogenic and antiatherogenic effects in animal models (reviewed by Bauman et al., 2006) and trans 10, cis 12 CLA, which is involved in fat metabolism and causes milk fat depression in dairy cows (Baumgard et al., 2000). Effects of supplementation with calcium salts of CLA on reproduction were first reported by Bernal- Santos et al. (20003), who observed a trend for decreased median days to first ovulation and an increase in percent of cows pregnant. At a similar level of CLA supplementation, Castañeda-Gutiérrez et al. (2005) also reported the same results, but effects were not observed at a higher dose. CLA isomers reduce prostaglandin synthesis in several system (see review by Belury, 2002), including rat reproductive tissues and ewe intercotyledonary endometrium (harris et al., 2001, Cheng et al., 2003). Thus, it is possible that CLA may improve reproductive variables through modulation of uterine prostaglandin production.

2.2.4 Fatty acid supplementation and follicular development

Fat supplementation can also influence ovarian follicular development by increasing cholesterol, the precursor of steroid hormones. It has been proposed that an increase in specific fatty acids like arachidonic acid in the follicular fluid could increase PGE production and stimulate steroidogenesis and ovulation (Abayasekara and Wathes,1999).A higher number of medium size follicles was observed in cows supplemented with soybean oil compared to cows fed a tallow and fish oil supplement (Thomas et al., 1997); HDL- cholesterol was increased for cows supplemented with soybeen oils and the authors hypothesized that accumulation of HDL-cholesterol in follicular fluid may haveeffected IGF-I concentrations thereby increasing follicular growth. Increased concentration of HDL- cholesterol in follicular fluid and a greater number of medium size follicles was also observed in cows receiving a diet containing

8% fat, where the main fatty acid were linoleic and palmitic acid (Wehram et al., 1991), granulose cels collected from these cows produced more pregneneolone and progesterone when incubated in vitro. The effect of lipoproteins on follicular development was further confirmed by Bao et al.,(1997); incubation with LDL-cholesterol stimulated proliferation of small luteal cells and progesterone production by large luteal cells independently from IGF-I production. However, progesterone was similarly stimulated with the in vitro addition of lipoproteins from cows fed fat as comparedto those from unsupplemented cows, so the effect of fat composition of the lipoprotein on progesterone synthesis is not definitive.

Cows supplemented with prilled fatty acids at 5% of DMI had increased plasma concentrations of cholesterol and progesterone on day 1 to 8 postinsemination (Carrol et al., 1990). Plasma concentrations of cholesterol, estradiol and progesterone also were increased in cows fed diets containg 5.2% fat where the predominant fatty acids were oleic and linoleic, but no effects on follicular fluid steroid concentrations or follicular fluid of cows supplemented with Ca salt of fatty acids containing palmitic and oleic acid. In addition, the authors analyzed fatty acid composition of follicular fluid and found that linoleic and oleic acid concentrations were negatively correlated to estradiol content in follicular fluid, whereas plasma linoleic acid concentration was positively correlated. The differences in fatty acid profile in follicular fluid suggest that fatty acids may have a physiological function in the follicle. Supplementation with linolenic acid increased the number of small follicles, but the number of medium and large follicles was unaffected and no differences were observed in plasma concentrations of progesterone (Ponte et al., 2006).In contrast, Oldick et al. (1997) reported greater plasma concentrations of progesterone when cows were supplemented with linseed oil that is high in linoleic acid. Increased circulating concentrations of progesterone may be caused by a decrease in hepatic clearance of progesterone when cows receive fat supplementation. Evidence for this was provided by Hawkins et al. (1995), who supplemented beef heifers with Ca salts of fatty acids and then removed the corpus luteum. They observed that progesterone clearance was slower in cows supplemented with Ca salts of fatty acids than in the control.

Furthermore, Sangsritavong et al. (2002) examined the clearance of estradiol and progesterone in non-lactating cows receiving soybean oil or no supplement; they observed that the half-life of both hormones was increased when soybean oil was infused. This effect was corroborated by the same aouthors in vitro; using liver slices incubated with progesterone and estradiol, the addition of linoleic acid to the media increased the half-life of progesterone and estradiol (Sangsritavong et al.,2002).

Burke et al.(1997) fed menhaden fishmeal on two large dairy farms, they observed improvements in pregnancy rate and increased progesterone concentration after PGF2α injection on one farm where milk production was increased and cows had more substantial loss in BCS.

Additionally, Juchem et al. (2002) found an interaction with season and early embryonic mortality in cows supplemented with Ca salts of ω-3 fatty acids; pregnancy rates at second insemination were greater for cows fed Ca salts of ω-3 fatty acids during the thermoneutral season and reduced during the heat stress season.

2.2.5 Effect of fatty acids on peroxime proliferator activated receptor

Polyunsatured fatty acids are regulators of gene expression that can impact lipid, carbohydrate and protein metabolism, as well as cell growth and differentiation (Jump, 2004). Therefore, it is possible that improvements in reproductive function may be partially mediated by regulation of nuclear transcription factors. PUFA are natural ligands for peroxime proliferator activated receptors (PPAR) and four subtypes of PPAR have been described: α,γ1,γ2, and δ. PPAR activation leads to the induction of many genes involved in fatty acid oxidation and storage, and inflammation including COX2 (Vanden Heuvel, 1999). Recently, PPARγ has been demonstrated to be present in ovarian theca and granulose cells (Komar et al.,2001) and to have a role in fertilization and embryo implantation in rodents (Cui et al.,2002). There is evidence of a role for PPARδ as a mediator of blastocyst implantation through regulation of the enzyme COX2 (Lim and Dey, 2000). However, to date it is not clear if dietary fatty acids alter reproductive outcome through modulation of PPAR expression.

2.2.6 Conclusion

Several experiments have measured hormonal responses with different fat supplements, but when designing experiments to evaluate effects of nutrition on reproduction a large number of cows are needed to ensure a reasonable probability of detecting small differences (Barton and Carrol, 1992). Perhaps as a consequence of this and the fact that rumen biohydrogenation alters post-ruminal supply of fatty acids, effects of fatty acid supplements on fertility are not conclusive. Interactions with other environmental and nutritional effects may also influence the outcome.

Furthermore, the effect of fat supplementation in fertility may be influenced in part by the extent of biohydrogenation of unsatured fatty acids occurring in the rumen; therefore, it is necessary to establish the exten of bypass of unsatured fatty acids of interest in each form of rumen protection. In addition, the mechanism of action through which dietary fat improves reproduction may differ among specific fatty acids, and according to the stage of lactation when they are supplemented. Understandin how CLA benefit milk production and reproduction may improve the opportunity to develop opltimal diets for each stage of lactation.

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Chapter 3

Effect of pasture on milk and cheese quality: characterization of traditional cheeses produced in Alta Irpinia (Campania, Southern Italy)

3.1 Introduction

There is increased consumer awareness that foods contain microcomponents that may have beneficial effect on health maintenance and disease prevention.

In milk fat these functional foods components include EPA (eicosapentaenoic acid), DHA (docosahexaenoic acid), and CLA (Conjugated Linoleic Acid). CLA is a potent anticarcinogen, and the National Academy of Science (National Research Council. 1989, Nutritional Requirements of Dairy Cattle) has pointed out that CLA is the only fatty acid (FA) that has been shown unequivocally to inhibit carcinogenesis in experimental animals. CLA is found predominately in food products from ruminant animals; milk and other dairy products are the major sources of CLA in the human diet (Ip C. et al., 1994; Parodi, 1999). The content of CLA in milk fat is affected by a number of factors including the diet. In particular, it is well known that, compared with non-grazing cows, grazing produce milk with higher proportion of CLA, vaccenic (VA) and linoleic acids (LN) (Dewhurst et al., 2006). This invests fresh forage with added value and highlights grazing as worthwhile natural production system. Nevertheless, the milk CLA proportions measured in cows at pasture are variable (0.5 to 1.7%) (Chillard et al., 2004). Furtheremore, pasture induces a modification of milk FA composition, which affects cheese texture (Chillard et al., 2004) and the sensory, physical, and manufacturing properties of dairy products (Kaylegian, 1995). Unsaturated FA rich milk produces less firm abundance cheeses (Martin et al., 2004). Pasture led to more “animal” and less “bitter” and less “sour” odour, such differences being less marked with pasteurized milk (Verdier-Metz et al., 2002a).

In Southern Italy, the dairy system in the hills and mountains is basically a semi-extensive system. In particular, in *Alta Irpinia*, a region of the Apennine Mountains in Campania region, the territory is entirely mountainous, and the climate is typical of Mediterranean mountains, with a wide daily and seasonal variation in temperature, the mean minimum temperatures ranging from 0.6 to 7.1 °C in winter (January), the mean maximum temperatures ranging from 16.0 to 27.2 °C in summer (August). The predominant land use is dairy farming on family-owned farms, often combined with dairy sheep farming. Dairy systems are based on pasture grazing, mostly available during spring and early summer, and two winter crop per year, generally grain (barley, wheat) and forage (oat, vetch, clover) crops. Usually animals are fed preserved forages and concentrates in winter and then are switched to pasture in spring. Milk produced from cow and ewe is used almost exclusively to make two typical cheeses, namely Caciocavallo and Pecorino. These cheeses, mainly produced at the artisanal level, have been a major factor in reinforcing the territorial and economic identity of this upland area of southern Italy and in maintaining the economic vitality of a traditional, low-density, grass-based form of semi extensive dairy.

Caciocavallo is one of the most typical pasta-filata cheeses of the Southern of Italy. The launch strategy of caciocavallo has been carried through the protected designation of origin (PDO) Caciocavallo Silano (EC No 1204/03). Nevertheless the origin of the raw material for producing Caciocavallo Silano is not strictly tied to a local environment as the set area includes five different regions of southern Italy, namely Campania, Basilicata, Calabria, Puglia and Molise. Moreover the cheese making process is not specific enough to produce well- defined product. As a result the PDO Caciocavallo Silano unified the different varieties but doesn't represent a single typical cheese. Although Alta Irpinia falls in the PDO area, many types of Caciocavallo not PDO labelled are produced in the area varying in raw material, starter culture, curd and cheese ripening conditions (Ercolini et al., 2008). Some efforts to valorize these productions have been made (e.g. the typical product certification *Caciocavallo irpino di grotta*, a kind of caciocavallo ripened in natural mountain caves) but other traditional and locally produced kinds of Caciovallo are valuable. The characterization of these cheeses could allow to differentiate them from similar products and to increase their market recognition.

The main objective of this study is to characterize traditional cheeses produced in Alta Irpinia in order to better addressing consumer needs and enhancing the competitiveness of these products. For those reasons the research focused on:

1) to survey caciocavallo irpino cheese-making procedures used at various farms and to develop a consensus description of the traditional procedures;

2) to verify pasture's influence on FA profile of milk and cheese;

3) to assess consumers' perception of any variability among cheeses made by different dairies and in different season.

3.2 Materials and methods

A preliminary survey was carried out during the 2009 to identify characteristics and cheese-making processing methods used for producing Caciocavallo cheese in Irpinia. A fifteen page survey form for data collection was developed and 21 farms were visited. The farmers traditionally made Caciocavallo cheese and ricotta daily from the milk produced on the farm. Cheese makers were almost always women who in this context make a large contribution to farm diversification. Caciocavallo cheese making generally took place in a room adjacent to the barn: the raw milk, in the main taken from two milkings and not thermally treated, was filtered and was coagulated with initial addition of rennet (36–38 C, 4.0 mg/kg) with 3% (v/v) natural whey culture (pH 3.80).

Commercial rennet solution from calf is generally used. Other farmers used rennet paste made from young goat, in particular to produce more piquant cheese. The natural and unselected whey starter was obtained by incubating the fresh whey, derived from a previous cheese making, at 40–42°C for about 24 h.

After ca. 30 min at 37–38 C, the coagulum is first cut coarsely by hand, heated under whey at 45°C for 2 h, reduced to particles of 1.5–2 cm and held at room temperature until the pH reaches a value of ca. 5.30. Thereafter curd was stretched until the typical flask-like shape of 1–2.5 kg sizes with a short neck and a small round top; after manufacture, the end of the body curd is tied with a cord. Stretching is carried out manually in hot water (70–80 °C). The cheeses were cooled in water, salted in brine (27–30% NaCl) for 6 h, air dried and ripened in a cool aging room hanged to a robust stick. Surveyed farms have different type of stockrooms: ancient or new building cellars with natural ventilation, and modern cellar with controlled temperature and humidity.

Cheeses were ripened for at least 1 month before to be sold, however, 3 months ripening age was mostly appreciated by consumers.

On the basis of information obtained from the preliminary survey, three dairy farms (A- B –C) were chosen for the study responding to the following criteria:

1) use of pasture as the primary source of feeding in spring and early summer;

2) farm Caciocavallo cheese-making by processing only self-produced milk;

3) cheese-making and ripening processes for production of caciocavallo typical of Alta Irpinia area.

Other relevant characteristics of farms are given in table 3.1 and 3.2.

The study was divided in two periods according the seasonal pattern of pasture exploitation. The first sampling period was in spring (i.e. from April 2009 to June 2009) during the grazing season. The second period was in winter (i.e. from December 2009 to February 2010), a time in which there were no pasture growth. Over each 3 month study period, farms were visited every two weeks, thus a total of twelve visits were made.

Table 3.1: Herd characteristics

Farm	Species	Cattle breed	Cow number
A	Cattle / sheep	Holstein Friesan Simmenthal	40
B	Cattle / sheep	Holstein Friesan Simmenthal	16
C	Cattle	Holstein Friesan Simmenthal	21

Table 3.2: Farm characteristics

Farm	UAA (ha)	Forage	Grain
A	80	Oat, Clover spp, Vetch	Barley, Oat, Wheat
B	26	Alfalfa, Oats, Clover	Barley, Wheat, Faba bean
C	25	Clover spp.	Wheat

3.2.1 Samples collection procedure

During grazing season the three herds were allowed to graze in fenced pastures in which areas of 1x1 m, randomly distributed, were delimited. The number of these areas was established according to the size of pastures (table 3.3). Each farm was visited in two consecutive days. On the first sampling day, four of the cows from the three herds were selected randomly for observation of their pasture grazing. Each cow was followed as closely as possible for a period of about 30 min and the plants selected were recorded and identified. On the basis of this information for each species, plant samples corresponding to those really browsed by cows were cut at 3 cm height from the ungrazed areas and mixed. This samples plus samples of feedstuffs used to fed cows in barns were collected and used for chemical analyses. On the second sampling day, bulk milk samples were collected, just before cheese making, refrigerated at 4° and sent to laboratory. Moreover, a caciocavallo cheese, weighing about 1.5 kg, produced from the milk sampled was identified and marked prior to the storage to the farm cheese-aging rooms for ripening. All cheese blocks were stored in farm cheese aging room for 120 d. Additional four cheese samples per each farm were identified and marked at the 3rd sampling time in order to assess sensory characteristics of cheeses from the three farms.

During the winter period, milk, caciocavallo cheese and feedstuffs samples were collected as previously described.

The cheese-making process and brining and ripening methods at each farm were documented approximately once each month throughout each sampling period.

Table 3.3: Pasture characteristics and number of pasture areas sampled

Farm	Pasture, ha	Sampled areas, n°	Pasture features
A	20	40	Natural meadow
B	13	26	Natural meadow, Clover spp., Ryegrass spp
C	3	6	Natural meadow, Clover spp., Ryegrass spp

3.2.2 Chemical Analyses

Pasture and feedstuffs

The AOAC (1990) official methods were used to determine DM, ash, Crude fiber (CF) and Crude protein (CP) (Nx6.25) contents in feedstuffs and pasture. Organic matter (OM) content was calculated as the difference between DM and ash contents. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) exclusive of residual ash were determined by methods of Van Soest et al. (1991), without the use of an amylase and sodium sulfite for NDF. Food samples were also analyzed for FA composition.

Milk and cheese:

The milk samples were split into two portions for analysis. One portion was immediately analyzed (AOAC, 1990) for fat, protein (N*6.38), lactose, and NFS by mid-infrared spectrophotometry (MilkoScan FT 6000, Foss Electric, Hillerød, Denmark); somatic cell count (SCC) were determined using a Fossomatic 90 (Foss Food Technology Corp.). Milk urea nitrogen (MUN) Milk urea nitrogen was determined using procedures described by Roseler et al. (1993). The remaining portion was stored at −20°C until analysis of FAs by Gas Chromatography. After 120 d of ageing, each marked caciocavallo cheese were removed from the ageing-rooms and sent to the lab. Cheeses were divided with a cutting machine in three parts (rind, under-rind and center) and each center part was divided in two half: one portion was analyzed for protein and ash. The remaining portion was used to extract fat and determine fatty acids profile by GC. The extraction of fatty acids from milk and cheese was done according to the method of Hara and Radin (1978). The methylation of fatty acids was performed by base-catalyzed transmethylation as described by Bernal-Santos et al. (2003). Fatty acid methyl esters were quantified by GC (Perkin Elmer Autosystem XL gas chromatograph) equipped with a fused silica capillary column SP 2380 (Supelco, Bellefonte, USA) 100 m x 0.25 mm i.d.; 0.20 mm film thickness, was used. The column was held at 100 °C for 5 min after injection, heated at 3 °C min-1to 165 °C, held at 165°C for 10 min, and then heated at 3 °C min-1to 260 °C and held at the final temperature for 28 min.

Fatty acid peaks in chromatograms were identified using pure methyl ester standards (Nu-Chek Prep., Inc. Elysian, MN, USA). A butter oil reference standard (CRM 164; Commission of the European Community Bureau of References, Brussels, Belgium) was also analyzes periodically to control for column performance and to facilitate the calculation of recoveries and correction factors for individual fatty acids.

3.2.3 Sensory properties of Caciocavallo cheese

Cheeses collected in spring and in winter in each farm, were sent to the Department of Animal Production at Università della Basilicata in Potenza (Basilicata region, Italy) to determine sensory properties.

Twelve panelists were trained for quantitative descriptive sensory analysis during several sessions in which descriptive terms were generated through group discussion for odor, taste, consistency, and appearance (color) attributes of caciocavallo cheese. Redundant terms were eliminated and consensus was developed for the meanings of the descriptors. By selecting terms with the highest correlation with differences in samples used in training, a single score card containing three odor, six taste, six consistency, and two color descriptors was developed representing the consensus profile of sensory characteristics (Table 3.4). Attributes were evaluated on 100-point intensity scales with labels at the left and right ends to indicate the direction of the scales. The panelists valuated five replications of cheese samples from the three herds (i.e. fifteen trials). In all tests, carried out at about 10.00 a.m., cheese cube samples (1 cm^3) were served in a randomized order. Tests were conducted in sensory rooms equipped with adequate lighting and individual booths. Red light was used during evaluation to mask color differences. The red light was turned off during color attributes evaluation. The samples were labeled with random three-digit code, and the panelists were not provided with information regarding each sample.

In order to avoid sensory fatigue due to the number of samples, in each session three samples were evaluated. The interval between samples was approximately 10 minutes. Between samples, panelists were instructed to rinse their mouths with a piece of apple. After the initial sessions, panel performance was assessed taking into account the following aspects:

(1) discriminatory ability (i.e) ability to notice differences between similar products differently made or with slight different characteristic (ie: farming system, cheese making process);

(2) reproducibility (ability of different panelist to have similar opinion after the same training);

(3) panel homogeneity.

Caciocavallo cheeses from the three herds and produced in the two periods were also evaluated for consumer acceptability

Flavor, texture, odor, and overall acceptability were evaluated by a 100 member untrained panel using a 9-point hedonic scale (9: like extremely to l: dislike extremely). The average score corresponds to products with good acceptability (Kähkönen et al., 1996).

3.2.3 Statistical analyses

Statistical analyses were performed by using SAS software (SAS Institute, 1990). Data of milk yield and quality were analyzed by one-way analysis of variance with period (i.e. winter and spring) and farm (i.e. A, B, C) as factors.

Data of fatty acid composition of milk and cheese were analyzed by analysis of variance for repeated measures using the Mixed procedure of SAS with sample (milk or cheese) as repeated factor and farm (A, B, C) and period as non repeated factor.

Sensory properties were analyzed by analysis of variance by using farm and period as main factors.

Table 3.4. Vocabulary of descriptors for Caciocavallo cheese

Smell/ flavor	Milk
	Butter
	Smoked
Taste	Acid
	Sweet
	Bitter
	Salty
	Piquant
	Aged
Texture	Tenderness
	Creaminess
	Graininess
	Adhesiveness
	Humidity
	Crumbliness
Aspect (Color)	Thickness
	Color intensity

3.3 Results

3.3.1 Botanical composition of pasture

The study was conducted under real condition of milk and Caciocavallo cheese production to cover the diversity of pasture area grazed. The mean botanical and chemical composition of the pasture of each farm was the average of samples and the number of samples collected varying depending on the time of pasture utilization. In total 18 samples were collected.

All grazing areas were characterized by a high proportion of grass (e.g. *Lolium* spp., *Avena* spp.,), but also contain many common Mediterranean plants (e.g., *Medicago hispida, Falaris tuberose, Agrostis canina and Trifolium subterraneum*), which may contribute desirable flavors to the milk and cheese. High percentages of non edible plants were found.

On the basis of the field observations, the different pastures were classified according the plants' species collected. We were able to recognize three different kinds of pasture:

- Natural meadow;
- Pasture with high incidence of Ryegrass spp.*;*
- Pasture with high incidence of Clover spp..

3.3.1.1 Natural meadow

This kind of pasture was observed in all farms. These pastures were mainly composed of grasses *(Hordeum vulgare*, *Avena sativa*, *Dactylis glomerata*, *Lolium multiflorum*), whereas legumes (*Trifolium* spp, *Medicago* spp.) were found in minor amounts. Moreover, high incidences of not edible and/or dangerous plants (e.g. *Ranunculus* spp., *Carduus* spp., *Convolvolus* spp., *Galium* spp., *Agrostis* spp*.)* were found.

Chemical and nutritional characteristics of pastures (i.e Crude protein, NDF. Milk FU/kg DM) varied throughout the observation period depending on vegetation growth stage. The high amount of NDF observed (on average 56.1$\pm$5.6) is related to the high incidence of grasses in the pastures.

3.3.1.2 Pasture with high incidence of Ryegrass

These of grazing areas, observed in the farms B and C, are periodically seed with *Lolium multiflorum*. Compared to natural meadows, these pastures showed lower incidence of not edible or dangerous plants. Moreover, lower CP and energy (Milk FU/kg DM) contents, and higher NDF percentages were observed, as these pastures presented reduced incidence of legume species (table 3.5). Pasture's quality was getting worse as the ryegrass vegetative phase progressed; in particular, NDF percentages and the incidence of not edible plants were rising throughout the sampling period.

3.3.1.3 Pasture with high incidence of Clover spp

These pastures, used in the farms B and C, include two variety of clover (*Trifolium squarrosum* e *Trifolium pratense*). The incidence of not edible plants was really low. As expected, these pastures showed higher CP and energy contents and lower CF and NDF percentages, compared to the kinds of pasture discussed earlier (table 3.5).

In conclusion, we can affirm that the pasture quality was very variable depending on plant species and plant different vegetative stage. Moreover pasture condition were quite poor due to:

- Use of continuous grazing system; in this system livestock are able to select the more palatable grasses and pastures tend to become dominated by unpalatable species. It could be useful to periodically rotate the animals on different areas.
- Fertilizers high in nitrogen are mostly used: these products are inappropriate to the growth of native and palatable plants in the native pasture.It would be better to use fertilizer rich in phosphorus
- Lack of weed control. Different strategies could be used to control weeds e.g. rotational grazing, winter grazing, cut or dig out weeds, etc.

3.3.2 Hay

Different hays (ryegrass, mixed, natural meadow, clover) were fed to cow during the survey periods (table 3. 6). Most of them showed high lignin percentages, reduced leafiness and mould occurrence. These characteristic negatively influenced the chemical composition of the hays.

The poor quality of hays was mainly due to the late harvesting time due to:

1) the farmer' tendency to produce high quantity of hay;
2) the climate of the area that usually not allow to harvest hay at right time.

3.3.3 Concentrated

Grains (wheat, oat, faba bean, etc) produced on farm and, in smaller quantity, commercial concentrates were used (table 3.7). The differences observed in chemical composition between the grains are mostly related to the different grains used and to their percentage in the final mixtures.

Table 3. 5:Characteristics of pastures

Natural meadow							
Farms	**Date of sampling**	**Ash**	**CP**	**CF**	**NDF**	**Milk FU/kg DM**	**Edible species ,%**
A	23-04	13.3	15.6	26.9	50.4	0.66	76
A	12-05	12.7	15.3	25.2	54.5	0.71	76
A	22-05	12.4	14.2	32.2	61.2	0.61	71
A	10-06	11.6	11.4	31.2	67.3	0.62	55
B	12-04	13.1	14.0	22.8	50.1	0.74	78
B	23-05	11.9	16.7	26.1	54.5	0.70	60
C	23-04	13.5	17.2	27.6	56.3	0.66	80
C	12-05	8.6	14.4	26.4	54.4	0.74	63

Ryegrass Pasture							
Farm	**Date of sampling**	**Ash**	**CP**	**CF**	**NDF**	**Milk FU/kg DM**	**Edible species ,%**
B	20-05	11.6	13.4	25.2	51.1	0.70	90
B	22-05	11.6	17.6	25.6	55.0	0.70	88
B	04-06	11.6	12.0	29.0	59.6	0.64	75
B	10-06	12.0	12.3	30.2	63.1	0.64	67
C	22-05	9.0	9.7	27.6	57.8	0.60	91
C	04-06	11.6	11.7	29.5	61.1	0.64	75

Clover Pasture							
Farm	**Date of sampling**	**Ash**	**CP**	**CF**	**NDF**	**Milk FU/kg DM**	**Edible species, %**
B	20-05	13.7	22.3	18.7	35.1	0.80	95
C	22-05	12.5	18.0	18.9	37.6	0.79	93
C	04-06	13.0	20.0	21.8	36.0	0.80	95

Table 3.6: Chemical characteristics of hays

Farm	**Hay**	**Ash**	**CP**	**CF**	**NDF**	**ADF**
B	Mixed grass	9.5	5.8	36.5	66.8	46.4
A	Mixed grass	9.2	7.1	35.0	63.9	42.0
C	Clover (*Tr. Pratensis*)	11.8	11.6	35.2	60.2	47.3
A	Clover (*Tr. Squarrosus*)	12.4	13.4	33.7	59.0	44.8
A	Natural meadow	8.1	6.3	36.4	65.6	47.2
A	Natural meadow	10.9	10.3	31.4	61.6	44.7
C	Natural meadow	11.4	7.1	29.4	57.0	39.5

Table 3.7: Chemical characteristics of concentrate

Farm	**Sample**	**Ashes**	**CP**	**CF**	**Starch**
A	1	2.8	10.3	5.9	56.9
A	2	1.6	9.1	1.9	68.9
B	1	2.5	9.6	3.5	64.4
B	2	2.3	10.9	3.2	59.4
C	1	2.7	10.4	4.5	59.1
C	2	2.3	10.8	3.5	57.4

3.3.4 Diets

Tables 3.8 and 3.9 show nutritional characteristics of the different diets used during winter and summer. Diets were more or less unbalanced as regard CP and energy content .

Table 3.8: Chemical and nutritional characteristics of diets (total and per kg of 4% fat correct milk, FCM) during spring.

Farm	DM	Milk FU	Milk FU /kg FCM	CP (g)	CP (%DM)	g CP /kg FCM	NDF (%DM)	Starch (%DM)
A	17	13.4	0.37	2182	12.8	79.3	45.0	14.1
B	17.2	13.5	0.42	2519	14.6	104.8	41.9	7.0
C	17.4	13.2	0.40	2135	12.3	83.9	46.7	13.5

Table 3.9: Chemical and nutritional characteristics of diets (total and per kg of 4% fat correct milk, FCM) during winter

Farm	DM	Milk FU	Milk FU /kg FCM	CP (g)	CP (%DM)	g CP /kg FCM	NDF (y)	Starch (%DM)
A	17.0	13.1	0.51	1600	12.1	72	57.2	20.4
B	16.2	12.9	0.57	1340	10.3	63	55.1	24.4
C	18.3	14.5	0.50	2400	12.9	105	41.6	24.5

3.3.6 Milk

3.3.6.1 Production and quality

Table 3.10 shows the milk yield in the two observation periods (spring and winter). Milk yield was rather low in all farms, with a slight improvement during spring. The low milk yield observed was due to several factors, the most important of which are the following:

1. presence of non-dairy breed cows;
2. poor farm management;
3. lack of milk yield recording systems.
4. unbalanced diets and poor quality of forages

The first two aspects are typical of dairy system of Irpinia area. As a consequence, the only factors that could be controlled are the last two.

Table 3.11 shows the milk chemical composition. The percentage of fat resulted slightly higher compared to average value of the Alta Irpinia area; on the other hand it should be noted that milk fat content is influenced by milk yield (i.e. dilution effect), cow age, cow days in milk and milking techniques.

Usually milk fat percentage increases when cows are switched to pasture as a result of the decline in milk production (Battaglini et al., 2001, 2003; Bianchi et al., 2002). Reduction in milk yield during grazing period was not observed in our study probably due to the fact that pasture feeding was integrated with forages and concentrates. Somatic cells count was often too high probably as a not correct milking technique or presence of subclinical mastitis. Anyway, milk from cows on pasture often has a higher somatic cells count compared to milk from cows in intensive system (in average < 400.000 cells/ml) (Agabriel et al., 2001; Coulon et al., 1997; Bugaud et al., 2001a; Pomiès et al., 2000).

Table 3.10: Milk yield and 4% FCM yield (kg/head/d)

Farm	Summer				Winter			
	Cow, n	Milk (kg/d)	Fat (%)	FCM (kg/d)	Cow, n	Milk (kg/d)	Fat (%)	FCM (kg/d)
A	20	21	4.03	21.1	22	15	4.26	15.6
B	10	21	3.42	19.2	16	13	4.07	13.1
C	20	20	3.79	19.4	21	17.6	3.68	16.8

Table 3.11. Milk traits in the three farms in spring and winter.

	Farm									
	A		**B**		**C**		**A+B+C**			**P**
	Season		**Season**		**Season**		**Season**			
	Spring	**Winter**	**Spring**	**Winter**	**Spring**	**Winter**	**Spring**	**Winter**	**ES**	
Fat %	4.03	4.26	3.42	4.07	3.79	3.68	3.56	3.98	0.134	*
Protein %	3.26	3.33	3.23	3.18	3.19	3.35	3.22	3.35	0.037	*
Lactose %	4.79	4.73	4.82	4.79	4.60	4.53	4.73	4.65	0.0347	NS
MUN mg/dl	24.78	14.03	10.53	18.63	18.46	13.70	19.29	16.82	1.98	NS
Cryoscopic index °C	-0.527	-0.530	-0.518	-0.525	-0.522	-0.535	-0.0527	-0.0532	0.0032	NS
Somatic cell count	309,750	436,500	485,600	406,750	655,800	926,000	505,108	639,986	72,143	NS

3.3.6.2 Milk fatty acid profile

Table 3.12 shows the acidic profile of milk produced in summer and in winter, i.e. with and without pasture allowance. Pooled data from the three farms are reported.

There were no significant differences between the FA profile of milk produced in two periods as regard short chain fatty acids (C≤15); only butyric acid (C4) and caprinic acid (C6), i.e. FAs related to milk and cheese flavor, showed higher values during grazing season. By contrast palmitic acid (C16:0) showed lower values in spring period. Linolenic acid (C18:3), CLA cis9-trans11 and polyunsaturated FA (PUFA) were higher in milk produced in spring.

The diet of the cow has a large impact on the fatty acid composition of the milk (Palmquist et al., 1993). Our data clearly show that grazing feeding was playing an important role on milk FA composition. Many studies have shown that increased pasture intake leads to elevations in the CLA and n-3 FA concentrations of milk (Kelly et al., 1998; Dhiman et al., 1999a; Kraft et al., 2003), potentially due to the higher concentrations of LNA in particular in fresh herbage compared with either conserved forages or cereal-based concentrate feeds (Schroeder et al., 2004; Dewhurst et al., 2006). This result might also be related to the high α-linolenic/ linoleic acid ratio of the animals' diet (Mel'uchová et al., 2008). Both fatty acids contribute either directly or indirectly in pasture for c9,t11 CLA biosynthesis. Grazing, compared with conserved forage and grain-based diets, increases ruminal pH and thereby favours the microbial production of CLA and VA, while the addition of grain and forage conservation techniques may decrease ruminal pH, which in turn negatively affects ruminal fermentation in respect to formation of CLA and VA (Dhiman et al., 1999a; French et al., 2000). Furthermore, seasonal effects on milk PUFA concentration have been identified, in which CLA was at a higher concentration in milk during spring and early summer compared with autumn, coincident with seasonal variation in the herbage PUFA content (Chilliard et al., 2001; Lock and Garnsworthy, 2003; Dewhurst et al., 2006). Our findings of milk fatty acid composition in spring compared with winter are in line with these observations.

When the milk FA content was analysed by farm (Table 3.13), the major effect of pasture allowance on CLA concentration was found for milk produced in farm A (+1.07%/weight), whereas the minor was for milk from farm C (+0.33%/weight). We argued that many of these differences could be explained by difference in pasture allowance in the three farms. In fact, the ratio between number of lactating cows and the hectares of pasture was rather different in the three farms: 2.0 vs 1.2 vs 0.7, respectively for farm A, B and C). According Palladino et al. (2009), the milk PUFA and CLA content in grazing cows can be influenced by the quantity and quality of the herbage available. Furthermore, reducing the daily herbage allowance reduced the concentration of CLA in milk (Stanton et al., 1997; Dewhurst et al., 2003). Additionally, Elgersma et al. (2004) recorded lower concentrations of milk CLA when herbage allowance was reduced by 50%. Despite this, other authors have failed to observe any effect of modifying the pasture allowance on milk PUFA content (Dewhurst et al., 2006).

As general consideration, the previous analysis indicate that milk product during the summer presents a better acidic profile from the nutritional point of view with less palmitic acid and larger amount of CLA and PUFA. However, PUFA could also negatively influence the cheese texture. In the sensory test it will be assessed if the different FA profile of cheese produced with or without pasture allowance have influenced sensory properties of cheese.

3.3.6.3 Caciocavallo cheese fatty acid profile

Table 3.14 shows the acidic profile of Caciocavallo cheese. Pooled data from the three farms were collectively analyzed. Overall, the FA profile of cheeses reflects the FA composition of raw milk used for cheese making with some differences due to the aging process.

In particular, compared to Caciocavallo cheeses produced without pasture allowance, CLA content of the cheeses produced from grazing herds increased more than fourfold (Table 3.14).

The analyses of acidic profile of cheese produced in the three farms (figure 3.1) reveals that the CLA contents of cheese produced without pasture allowance in the farms A and B was rather small and lower than the values observed in the raw milk used for cheese making. Nevertheless, in the cheeses produced in spring the CLA content raised to values comparable to those observed in raw milk produced in the same period. By contrast, the cheeses produced in the farm C had did not show any differences between the seasons, and the value observed was intermediate compared to those of raw milk with or without pasture allowance.

The major effect of grazing feeding on cheese CLA concentration was found for milk produced in farm A (+0.93%/weight), closely followed by the difference observed in farm B (+0.86%/weight). Overall CLA concentration in cheeses and milk showed different patterns in the three farms.

Characteristics of ripened cheese depend largely on technological factors and consequently it is difficult to ascertain and interpret the effects of upstream factors, such as feeding. The effects of processing conditions, storage, and aging on the CLA content of various types of cheese are not very clear and reports and reviews present results for individual varieties. These effects are likely to be small, and variations in CLA levels are similar to the levels in the starting milk (Dhiman et al, 1999b; Gnädig, et al., 2004; Ryhänen, et al., 2005). However, other studies have detected changes in the CLA levels or new isomers in ripened cheeses (Werner et al., 1992; Lin et al., 1999; Luna et al., 2005). Moreover, some authors found that CLA content of cheese fat varied with processing temperature (Shantha et al., 1992) and was higher in cheese with a long aging time (Zlatanos et al., 2002).

In this study the cheeses from the three farms were made in similar way as described above, and were aged for the same time. On the other hand, Caciocavallo cheese is obtained by coagulation of whole raw cow milk, at a natural pH, to which natural whey starter is added. The natural whey starter is a culture of bacteria, obtained from whey of the previous day's cheese-making and incubated at room temperature (Villani et al., 1991). As the LAB microflora arising from the raw milk or the natural starter cultures used in cheese manufacture is dairy linked (Gatti et al., 2003; Piraino et al., 2003), we hypothesize that in farm A and B biohydrogenation activity of microflora from raw milk or natural whey led to decrease CLA concentration in cheese.

Table 3.12: Milk fatty acid profile (%/weight) produced in Winter and Spring

Fatty Acid	Winter	Pasture	P
c4	2.47	3.18	*
c6	1.41	1.67	*
c8	0.98	1.07	ns
c10	2.56	2.73	ns
c12	3.41	3.29	ns
c14:0	11.9	1189	ns
c16	33.4	29.4	***
c18	11.51	12.54	ns
c18:1 n9c	21.53	22.1	ns
c18:2n6c	2.38	2.10	*
c18:3n3	0.65	0.85	**
CLA	0.37	0.99	***
de novo synthesis FAC≤15	25.4	26.4	ns
Mixed origin: C:16:0 and C16:1	35.9	31.9	***
Preformed FA: C≥17	38.7	41.7	*
Saturated FA	70.9	69.0	ns
MUFA	25.4	26.6	ns
PUFA	3.76	4.34	**

Table 3.13: Milk fatty acid profile (%/weight) produced in the three farms in winter and spring

FATTY ACID	FARM		
	A	B	C
C4			
Winter	2.27	2.95	2.20
Spring	2.86	2.73	3.96
C6			
Winter	1.35	1.63	1.26
Spring	1.49	1.42	2.09
C16			
Winter	31.46	31.9	33.7
Spring	31.9	26.2	30.0
C18:3			
W/out pasture	0.70	0.57	0.66
with pasture	1.11	0.76	0.66
CLA			
Winter	0.38	0.40	0.32
Spring	1.4	0.91	0.65
PUFA			
Winter	3.26	3.80	3.80
Spring	4.80	4.02	4.27

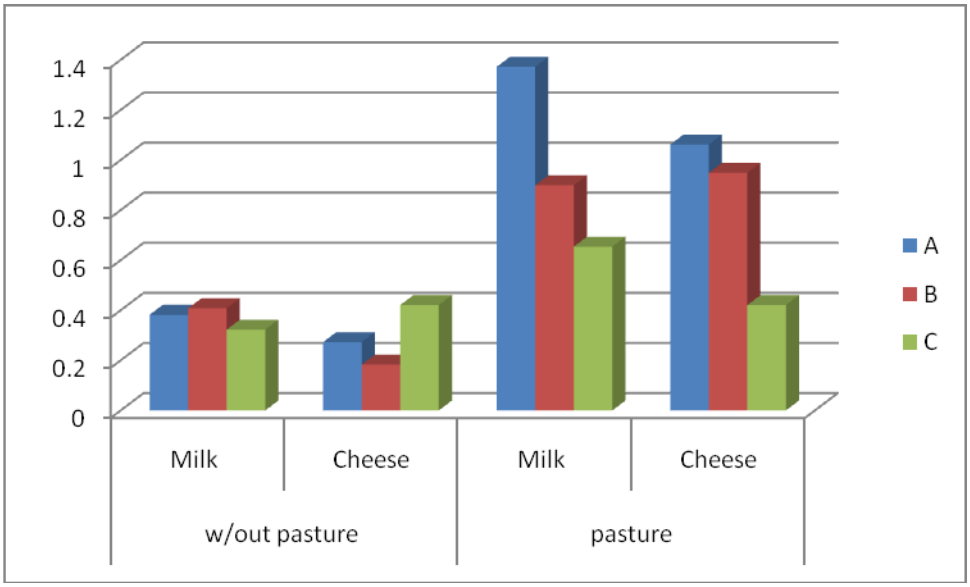
Table 3.14. Caciocavallo cheese fatty acid profile (%/weight) produced with or without pasture allowance.

FATTY ACID	Winter	Spring	P
c4	2.720	2.740	ns
c6	1.300	1.610	*
c8	0.920	1.110	*
c10	2.688	2.940	ns
c14	12.630	12.010	ns
c141	0.917	0.870	ns
c16	35.670	30.240	**
c16:1	2.440	2.290	ns
c18	10.270	12.000	*
c18:1t	0.420	0.500	*
c18:1 n9c	19.500	21.620	*
c18:2n6c	2.380	2.240	ns
c18.3n3	0.530	0.804	*
CLA	0.193	0.838	*
de novo synthesis FAC≤15	26.720	27.260	ns
Mixed origin: C:16:0 and C16:1	38.110	32.540	**
Preformed FA: C≥17	35.157	40.200	*
Saturated FA	73.140	70.140	T
MUFA	23.450	25.540	ns
PUFA	3.400	4.310	*

Table 3.15. Caciocavallo cheese fatty acid profile (%/weight) produced in the three farms in winter and spring.

FATTY ACID	FARM		
	A	B	C
C6			
Winter	1.5	1.0	0.6
Spring	1.6	1.33	1.98
C8			
Winter	1.04	1.10	0.63
Spring	1.38	0.97	0.33
C16			
Winter	36.18	35.19	35.66
Spring	30.5	29.12	31.11
C18:3			
Winter	0.49	0.59	0.51
Spring	1.00	0.77	0.63
CLA			
Winter	0.16	0.13	0.42
Spring	1.09	0.99	0.42
PUFA			
Winter	3.01	3.29	3.9
Spring	4.41	4.12	4.41

Figure 3.1 CLA concentrations in milk and cheese in winter and spring (with or without pasture allowance)



3.7 Sensory test

The interactions farm*replicate were never significant. This result is especially encouraging, since indicates that any cheese was evenly judged at various replications.

Significant interactions between farm and period were observed for most of the variables measured, reflecting the fact that the season of production had different effects on sensory properties of cheeses from different farm (table 3.16).

A significant effect of farm was found for all the attributes related to aspect (color), smell/flavor (except butter and smoked), taste, and texture (except adhesiveness) (table 3.16; figure 3.2).

Compared to the cheeses from farms B and C, the Caciocavallo cheese from farm A showed significantly lower values for smell/flavor (milk), taste (sweet), aspect (color intensity) and texture (adhesiveness, humidity, creaminess, tenderness), whereas presented higher scores for crumbliness and graininess (texture), aging, piquant, bitter and salty (taste).

The Caciocavallo cheese from the farm B resulted thicker and with a more intense yellow color. The Caciocavallo cheese from the farm C showed intermediate characteristics, but it appears closer the cheese from the farm B (Figure 3.2).

Significant effects of sampling period (Spring vs Winter) were observed for smell/flavor (butter and smoked), taste (bitter, piquant, ageing), aspect (color intensity) and texture (graininess, crumbliness).

The higher color intensity (yellow) scores observed in pasture cheeses indicate that compounds (usually β-carotene and related carotenoid compounds) present in fresh plant materials transferred from diet to cheese. β-carotene in milk depends directly on β-carotene content of forages. Indeed, as β-carotene is highly sensitive to ultraviolet light (Park et al., 1983), it is degraded during grass drying and preservation proportionally to the degree of light exposure. The type of diet therefore has a marked effect on carotene content in milk, hence on the color of butter and cheeses (Houssin et al., 2002; Martin et al., 2005b). So, cattle dairy products made with spring milk are much more yellow than those made with winter milk. In preserved diets, dairy products made with milk arising from grass silage are far more yellow than those made with a hay milk, especially when the hay was left on the ground for a long time (Martin et al., 2005a).

Our results confirm the results of several researches in which sensory differences have been observed between cheese from cows given winter diets (based on hay and grass silage) or turned out to pasture in the spring. Saint-Nectaire cheeses made with pasture milk were more yellow, with a less firm texture, stronger taste and less piquant, less sour and less fruity flavour than those made with winter milk (Verdier-Metz et al., 2000b). Similar results were observed by Buchin et al. (1998) and Verdier-Metz et al. (2002a) in trials that compared, respectively, semi-hard (Morbier) or hard (Cantal) cheeses made with milk from cows fed hay based diets or spring pasture. Carpino et al. (2004) showed that the pasture addition to a maize silage based diet resulted in Ragusano cheeses more yellow, less difficult to fracture and with higher score for floral and herbaceous odours.

In the previous section was observed that cheeses from pasture fed cows presented higher PUFA and long chain FA. This effect was due to the fact that fat from pasture, in comparison with that from preserved diets, has a higher proportion of unsaturated FAs and a lower proportion of saturated fatty acids (Chilliard et al., 2001). The lower melting point of unsaturated fatty acids produces a more fluid fat and consequently a more spreadable butter and softer cheeses (Martin et al., 2005).

However, in this study no differences were observed between winter and spring cheeses for tenderness, adhesiveness and creaminess. The only sensory attributes related to texture that significantly differed between winter and spring cheeses were crumbliness and graininess as the results of the higher values observed for these attributes in winter caciocavallo cheeses from farm A. We concluded that the different FA profile of cheese produced with or without pasture allowance did not really affect the texture of cheeses.

Table 3.16: Caciocavallo cheese descriptors: aspect/color, smell/ flavor, taste (Means ±SE)

	Summer Farm			Winter Farm			Significance		
	A	B	C	A	B	C	Farm	period	Farm*period
				Aspect/color					
Thickness	20. 5± 2.79	67.47 ± 2.79	57.11 ± 2.79	48. 13± 2.79	30.31 ± 2.79	66.62 ± 2.79	***	NS	***
Color intensity	35.91 ± 2.92	68.20 ± 2.92	42.04 ± 2.92	39.37 ± 2.92	43.58 ± 2.92	34.09 ± 2.92	***	***	***
				Smell/Flavor					
Milk	35.62 ± 3.99	47.42 ± 3.99	47.40 ± 3.99	40.20 ± 3.99	32.60 ± 3.99	41.71 ± 3.99	NS	0,10	*
Butter	28.18 ± 3.60	30.38 ± 3.60	22.44 ± 3.60	32.29 ± 3.60	36.96 ± 3.60	29.40 ± 3.60	*	*	NS
Smoked	5.60 ± 2.84	14.96 ± 2.84	10.22 ± 2.84	10.67 ± 2.84	27.53 ± 2.84	14.82 ± 2.84	***	**	NS
				Taste					
Sweet	14.67 ± 3.90	44.04 ± 3.90	43.31 ± 3.90	34.47 ± 3.90	28.24 ± 3.90	25.76 ± 3.90	**	NS	***
Salty	44.98 ± 2.95	19.58 ± 2.95	20.13 ± 2.95	23.51 ± 2.95	24.91 ± 2.95	25.40 ± 2.95	***	NS	***
Acid	19.73 ± 2.45	10.71 ± 2.45	11.84 ± 2.45	15.36 ± 2.45	20.15 ± 2.45	13.93 ± 2.45	NS	NS	*
Bitter	21.80 ± 2.73	12.78 ± 2.73	9.20 ± 2.73	16.76 ± 2.73	25.18 ± 2.73	22.04 ± 2.73	NS	**	**
Piquant	40.02 ± 2.40	10.71 ± 2.40	12.91 ± 2.40	13.18 ± 2.40	21.02 ± 2.40	11.04 ± 2.40	***	**	***
Ageing	57.44 ± 2.90	25.71 ± 2.90	32.31 ± 2.90	34.40 ± 2.90	27.84 ± 2.90	41.11 ± 2.90	***	*	***

*** = P<0.001; ** = P<0.01; * = P<0.05; NS = not significant

Table 3.16: Caciocavallo cheese descriptors: texture (Means ±SE)

	Summer Farm			Winter Farm			Significance		
	A	B	C	A	B	C	Farm	period	Farm*period
				Texture					
Tenderness	31.78 ± 3.57	64.44 ± 3.57	53.29 ± 3.57	45.24 ± 3.57	56.44 ± 3.57	39.69 ± 3.57	***	NS	***
Crumbliness	64.11 ± 3.04	19.82 ± 3.04	24.93 ± 3.04	24.60 ± 3.04	17.69 ± 3.04	32.42 ± 3.04	***	***	***
Graininess	65.58 ± 3.22	17.57 ± 3.22	24.22 ± 3.22	26.38 ± 3.22	13.56 ± 3.22	37.35 ± 3.22	***	***	***
Adhesiveness	42.09 ± 3.40	35.16 ± 3.40	38.09 ± 3.40	34.02 ± 3.40	34.91 ± 3.40	35.98 ± 3.40	NS	NS	NS
Creaminess	16.93 ± 3.07	37.98 ± 3.07	35.67 ± 3.07	25.44 ± 3.07	36.91 ± 3.07	20.48 ± 3.07	***	NS	***
Humidity	27.02 ± 3.32	39.89 ± 3.32	34.73 ± 3.32	32.97 ± 3.32	47.00 ± 3.32	29.33 ± 3.32	***	NS	NS

*** = P<0.001; ** = P<0.01; * = P<0.05; NS = not significant

Figure 3.2: Spider plot of sensory profile of the Caciocavallo cheeses produced in the three farms in spring.

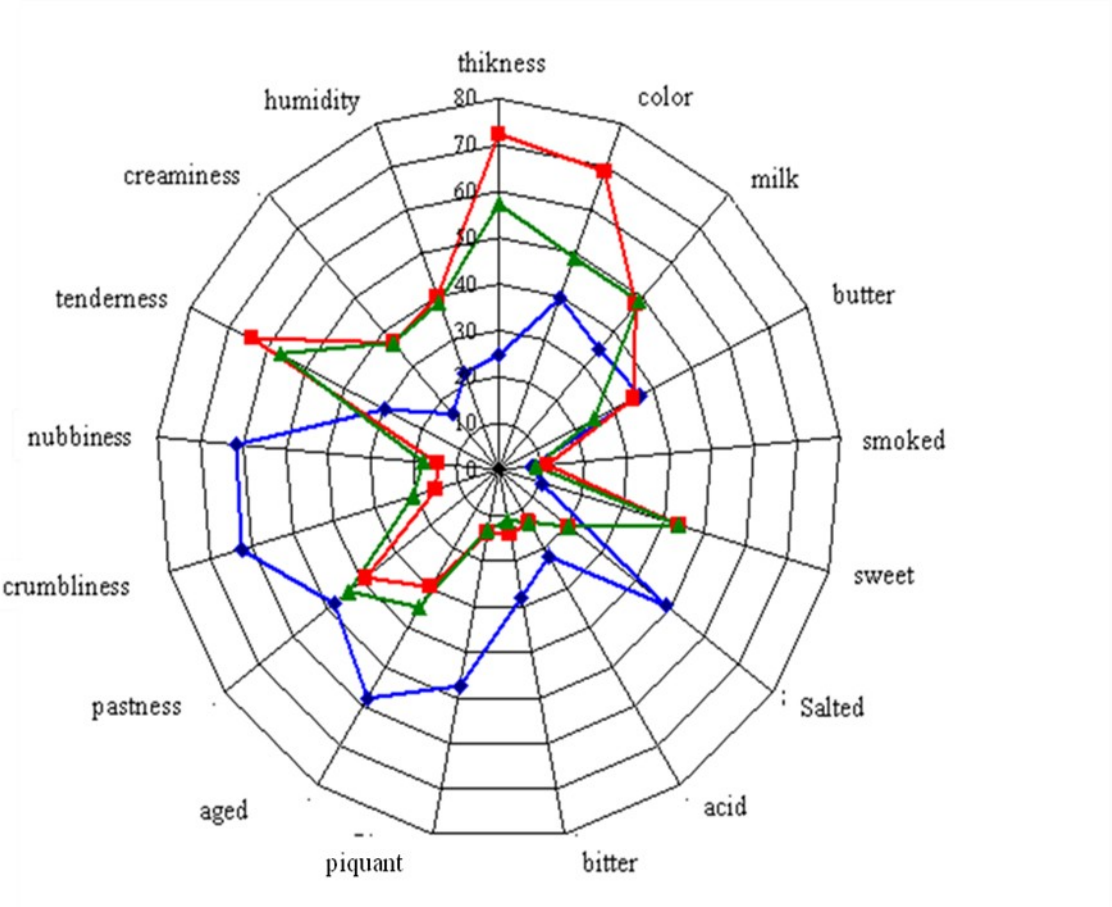
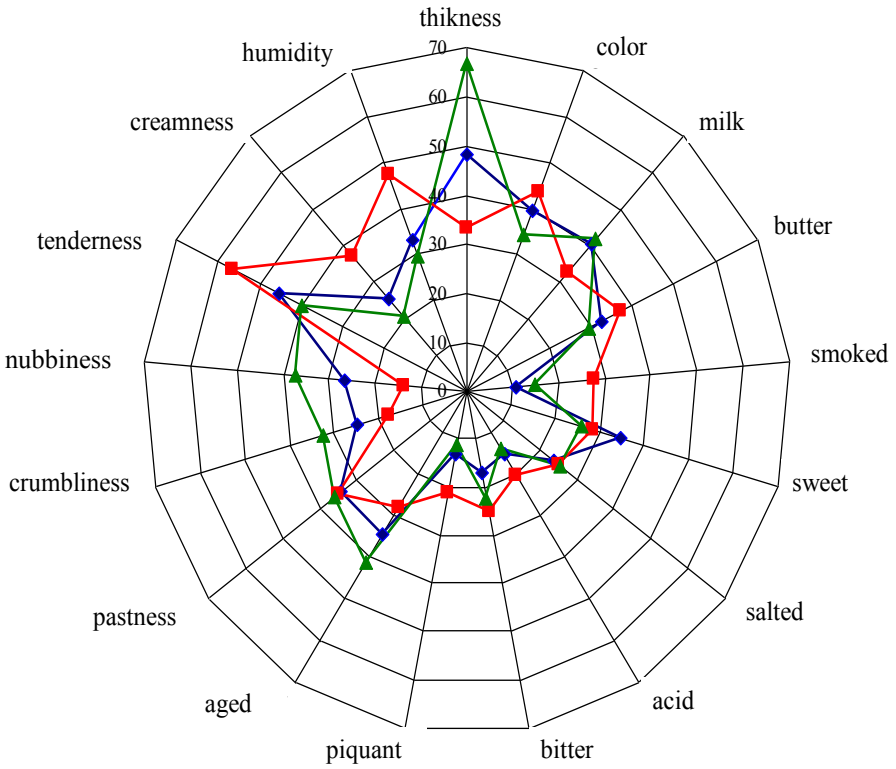
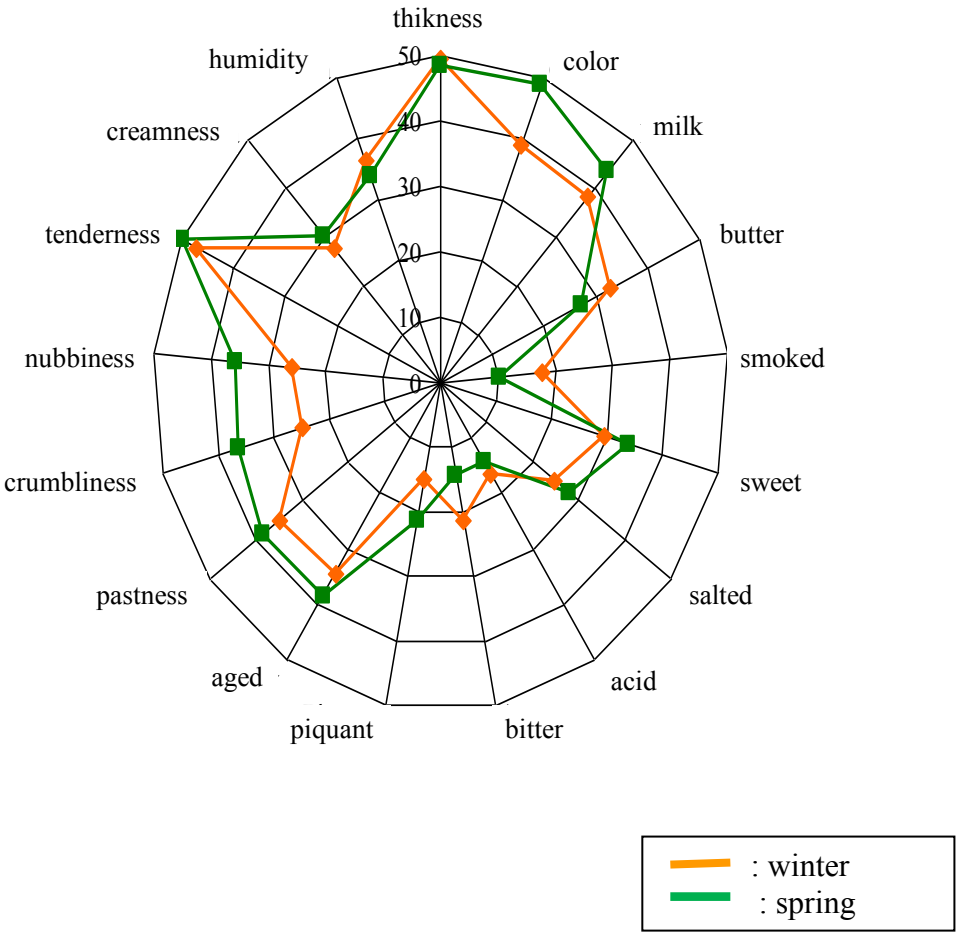


Figure 3.3: Spider plot of sensory profile of the Caciocavallo cheeses produced in the three farms in winter.



BLUE line: - caciocavallo cheese farm A
RED line: -:caciocavallo cheese farm B
GREEN line: - caciocavallo cheese farm C

Figure 3.4: Spider plot of sensory profile of the Caciocavallo cheeses produced in winter and spring.



3.8. Affective testing

Table 3.20 show that the panelists liked all the cheeses since the average scores were always above 5 (neutral value). However, cheeses from farm B were liked less than the others. The pasture allowance didn't make any difference about the taste of the products. Probably, this is due to the fact that in the two periods the Caciocavallo cheeses were made in the same farms, by the same people, by using the same cheese-making process. Moreover, during the cheese making process, the stretching phase is carried out in hot water (70–80 °C), modifying the sensory properties of so that the untrained panelists were not anymore able to detect differences between the cheese.

Table 3.20: Acceptance (mean ± S.E.) of caciocavallo cheese

	Spring			Winter			Significance	
	A	**B**	**C**	**A**	**B**	**CCII**	farm	Period
Acceptance	6,97 ± 0,17	6,56 ± 0,17	6,98 ± 0,17	6,78 ± 0,17	6,59 ± 0,17	6,67 ± 0,17	NS	NS
Aspect	7,10 ± 0,16	6,75 ± 0,16	6,82 ± 0,16	6,74 ± 0,16	6,66 ± 0,16	6,79 ± 0,16	NS	NS
Smell	6,78 ± 0,16	6,18 ± 0,16	6,68 ± 0,16	6,82 ± 0,16	6,28 ± 0,16	6,66 ± 0,16	0,10	NS
Texture	7,07 ± 0,15	6,66 ± 0,15	6,85 ± 0,15	6,95 ± 0,15	6,64 ± 0,16	6,73 ± 0,15	NS	NS

NS= not significant

3.9 Conclusions

This research focused on three main feature of dairy farm system of Alta Irpinia area:

1. Farm management;
2. Milk yield and quality;
3. Caciocavallo cheese quality

In the farms surveyed, cow' requirements were seldom met due to the poor quality of the forages, the not-rational use and exploiting of natural resources and the inadequate lactating cow management. Essentially the farms in this area need to be supported by technical assistance services that could reduce the production costs and improve the milk productions.

Pasture management and improvement really represent a critical point that may lead negative or positive consequences on the economic vitality of the territory and product quality. Some practical advices for pasture management are related to weed control, rotational grazing, use of fertilizers, and introduction of herbs. However to improve the pasture quality, it's needed to take in account the geological and climatic characteristics of the area and to act by little inputs to avoid loosing of efforts in a not really responsive territory.

The problem highlighted about milk production is, mainly, still related to an incorrect livestock management. As regard milk quality, beside the somatic cells count, it resulted satisfying. The higher count of somatic cells, probably due to the poor hygienic conditions during the milking process, can affect milk yield and quality.

As regard cheese quality, pasture feeding positively influenced the acidic profile of the Caciocavallo cheeses that presented higher level of CLA and PUFA, higher percentage of butyric acid and lower content of palmitic acid. This result represents a straight for Alta Irpina territory as it could allow to improve the profitability of dairy farms and also to preserve and to enhance the pastures areas.

As already said, from the sensory test the only differences noticed were related to the cheese making farm more than to the feeding. These differences also if not really strong, don't allow the characterization of the products.

In conclusion, it can be affirmed that the dairy system of Alta Irpinia area, if technically supported, has a high potential for improvement.

Overall our results constitute objective data for dairy farmer of Alta Irpinia area, as they refer back to the measures to be taken to update or maintain certain feeding conditions (i.e. maintenance of pasture) so that dairy products best reflect the uniqueness and diversity of the native land where they are produced.

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Chapter 4

Effect of dietary CLA on reproduction and metabolism in dairy cows

4.1 Introduction

Intensive genetic selection for increased milk production, coupled with technological improvements in nutrition, has led to significant increases in milk yield in dairy cows in recent decades (Taylor et al., 2004). However, this increase in milk output per cow has been accompanied by a worldwide decline in cow fertility (MacMillan et al., 1996). The decline in pregnancy rate to a single artificial insemination has been reported to be approximately 0.45%–1% per annum (Royal et al., 2000; Butler et al., 2003). High-yielding dairy cows are typically in a state of negative energy balance postpartum because the amount of energy required for maintenance both of metabolic function and milk production exceeds the amount of energy cows consume. Insufficient energy supply results in poor reproductive performance, which includes a delay in the onset of estrous cycles postpartum (Butler and Smith, 1989; Reist et al., 2000) resulting in low conception rates and a high rate of early embryonic death (Lucy, 2001).

Dietary fat supplements in early lactation may benefit reproductive outcome by improving energy intake and reducing the extent of negative energy balance, as well as by increasing size of the ovulatory follicle and lifespan of the corpus luteum (Mattos et al., 2000)

Furthermore, it was shown that in a diet with CLA supplementation the plasma concentrations of Insulin-like growth factor-I (IGF-I) is elevated (Castaneda-Gutierrez et al., 2007) and reported that cows with greater circulating IGF-I during the first 12 wk postpartum were more likely to conceive than those with lesser IGF-I. The mechanism through which CLA increased circulating IGF-I is unknown, but it was associated with the mixture that provided larger amounts of *trans*-10, *cis*-12 CLA (CLA 50:50). During negative energy balance in early lactation, the liver is refractory to growth hormone (GH) resulting in low concentrations of circulating IGF-I, but greater insulin availability restores coupling of the GH-IGF-I axis increasing circulating IGF-I concentrations (Butler et al., 2003). In other studies a 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA increased insulin sensitivity in muscle (Ryder et al., 2001)and increased genes related to insulin sensitivity in Zucker diabetic rats (Inoue et al., 2006).

Also the mechanism by which the granulosa cells regulate uptake of PUFA from plasma has not been elucidated. Offer et al. (Offer et al., 2001) found that concentration of PUFA were 10 times greater in the high density lipoproteins (HDL) and low density lipoproteins (LDL) fractions of plasma than in the very low density lipoproteins VLDL fraction and the granulose cells are able to use LDL to produce progesterone (P4) (Senger, 2003). (Lohrke et al., 1998) also shown that in the lutein cells, the peroxisome proliferator-activated receptor γ (PPARγ) is involved in regulation of P4 secretion and in the maintenance of a quiescent differentiated stage of lutein cells.

The functional capability (ability to produce progesterone) of the newly developed corpus luteum may depend on the degree of vascularity in the cellular layers of the follicle. The ability of the corpus luteum to vascularize may relate to its ability to synthesize and deliver hormones. The degree to which these angiogenic factors promote vascularization of the corpus luteum is probably related to the quantity of angiogenic factors present in the follicular fluid (Senger, 2003).

The main objective of this study is to understand better how CLA physiologically affect fertility in cows, focusing on the possible mechanisms involved:

1: IGF-I is produced primarily by the liver as an endocrine hormone as well as in target tissues in a paracrine/autocrine fashion. Production is stimulated by GH and can be retarded by undernutrition, growth hormone insensitivity, lack of growth hormone receptors, or failures of the downstream signaling pathway post GH receptor including SHP2 and STAT5b. Approximately 98% of IGF-I is always bound to one of 6 binding proteins (IGF-BP). IGFBP-3, the most abundant protein, accounts for 80% of all IGF binding. IGF-I binds to IGFBP-3 in a 1:1 molar ratio. Analyzing the GH receptors in liver and the IGF-I in the liver and in the Corpus Luteum (CL), will allow to understand if the improvement of the IGF-I by the CLA is because they either act improving the GH concentration or act in the liver.

2: Analyzing plasma and follicular fluid for Cholesterol, HDL, LDL; analyzing granulose cell's membrane for the LDL's receptors will allow to understand if the CLA, modifying the plasma level of the lipoproteins can affect the steredogenesis acting in the granulosa cells.

Analyzing all these factors will allow to understand if the ability of the CLAs to improve fertility in cows is due to local or systemic action.

4.2 Materials and Methods

4.2.1 Animals, Diet and Experimental Design

Multiparous Holstein cows (n= 24), from Teaching and Research Barn of Cornell University (Ithaca, NY, USA),15 days before the predicted calving date (d -15), were blocked by parity, body weight (BW) and body condition score (BCS) and were assigned in a randomized design to two treatments: a control diet and a "CLA diet" added with a rumen-protected formulation of *cis-9,trans-11* and *trans-10, cis-12* isomers of Conjugated Linoleic Acid *(Lutrell*Pure©;* table 4.1).The CLA were provided by BASF- The Chemical Company (BASF SE, G-EMN/MD - LI554, D-67117 Limburgerhof, Germany). The supplement was top-dressed on the Total Mixed Ratio (TMR) once daily. The supplementation was provided from d-15 to the end of the experiment set at 65 days in milk (DIM). All cows were fed with a close-up diet from day -15 to day 0; with an early lactation diet from 1 to DIM 19 and with a lactation diet from DIM 20 to the end of the experiment (table 4.2).

Table 4.1: Characteristic of rumen-protected CLA

CAS Number	Content W/W	Chemical Name
	42.5 %	Vegetable fat
1343-98-2	27.5 %	Silicic acid
112-62-9	11.9 %	methyl oleate
13058-52-1	9.52 %	9,11-Octadecadienoic acid, methyl ester, (9Z,11E)-
21870-97-3	9.52 %	10,12-Octadecadienoic acid, methyl ester, (10E,12Z)-
112-61-8	3.06 %	Octadecanoic acid, methyl ester
112-39-0	3.06 %	Hexadecanoic acid, methyl ester

The basal diet was a TMR formulated using the Cornell Net Carbohydrate and Protein System (Fox et al., 2004) to meet or exceed nutrient requirements (table 4.2). The TMR was sampled weekly. The TMR Dry Matter (DM) content was immediately determined by drying at 54°C until constant weight, and then samples were ground and composited at 4-wk intervals. Feed composites were analyzed by wet chemistry methods for CP, ADF, NDF, and either extract (Dairy OneCooperative Inc., Ithaca, NY, USA). Chemical composition of the diets are shown in table 4.3; 4.4; 4.5.

Cows were fed ad libitum to allow 10% orts. Individual daily DM intake (DMI) were record throughout the treatment period. Water and mineral blocks were always available. BW and BCS (5-point system; (Wildman et al., 1982)) were recorded every four days. From 26 DIM all the animals were synchronized by an injection (25

mg) of prostaglandin F 2α (PGF2α) (Lutalyse; Pharmaca and Upjohn) and on the same day, a progesterone releasing implant were inserted intravaginally (Controlled Internal Drug Release [CIDR] device, InterAg, Hamilton, New Zeland); after one week, on day 33 it was removed and the cows received a second PGF2α injection.

Cows were milked 2 times per day and milk production was recorded electronically. Every ten days, composite milk samples were collected, and stored at 4°C with a preservative (2-bromo-2nitropropane-1,3-diol: Bronolab-W II) until analyzed for fat, true protein, somatic cells, and lactose as described by Bernal-Santos et al.(Bernal-Santos et al., 2003). Somatic cell count (SCC) were determined using a Fossomatic 90 (Foss Food Technology Corp.). A second aliquot was stored without preservative at – 20°C until analyzed for fatty acids.

Table 4.2: Characteristics of the Closeup Diet (dry cows); early lactation diet, Lactation Diet.

Closeup Diet		
Ingredients	**As Fed, Kg**	**DM, kg**
Wheat Straw	1.6	1.4
Haylage	3.94	1.35
Corn Silage	12.43	4.85
Hay	1.47	1.25
Total forage	19.44	8.86
HR5 Closeup M066140 CornTR	4.33	3.86
Total concentrate	4.33	3.86
Total ration	23.77	12.72

early lactation diet		
Ingredients	**As Fed, Kg**	**DM, kg**
Wheat Straw	0.52	0.45
Haylage	7.9	3.61
Corn Silage	24.84	8.69
Total forage	33.24	12.77
Corn- Fine groud	1.13	0.97
DD Grins-WNY Energy	0.56	0.5
High Focus 08170 CornTR	9.03	8.03
Total concentrate	10.72	9.50
Total ration	43.96	22.27

Lactation diet Ingredients	As Fed, Kg	DM, kg
Haylage	8.7	3.97
Corn Silge	37.22	10.39
Total forage	41.34	14.37
Corn- Fine groud	3.03	2.60
Fat- Energy Booster 100	0.13	0.12
DD Grins-WNY Energy	1.23	1.1
HR1 Dairy, M07230CornTR	2.31	2.06
High Focus M07290 Corn TR	6.85	6.10
Total concentrate	12.31	11.98
Total ration	53.65	26.35

Table 4.3: Nutritional characteristic of the Closeup Diet

Components	As feed	DM
% Moisture	9	
% Dry Matter	91	
% Crude Protein	13.2	14.5
% Available Protein	2.6	3.9
% Adjusted Crude Protein	13.2	14.5
% Acid Detergent Fiber	23.8	26.2
% Neutral Detergent Fiber	37.9	41.7
% TDN	62	68
NEL, (mcal/kg)	1.46	1.61
NEM, (mcal/kg)	1.44	1.58
NEG, (mcal/kg)	0.89	0.98
% Calcium	1.16	1.27
% Phosphorus	0.34	0.37
% Magnesium	0.34	0.37
% Potassium	1.35	1.49
% Sodium	0.107	0.118
PPM Iron	294	324
PPM Zinc	72	79
PPM Copper	12	13
PPM Manganese	60	66
PPM Molybdenum	<1	<1

Table 4.4: Nutritional characteristic of the early lactation diet

Components	**As Fed**	**DM**
% Moisture	9.2	
% Dry Matter	90.8	
% Crude Protein (CP)	15	16.5
% Available Protein	14.5	16
% Acid Detergent Insoluble CP	0.5	0.6
% Adjusted Crude Protein	15	16.5
% Acid Detergent Fiber	17.4	19.1
% Neutral Detergent Fiber	31.9	35.1
% TDN	64	70
NEL, (mcal/kg)	1.5	1.66
NEM, (mcal/kg)	1.51	1.66
NEG, (mcal/kg)	0.96	1.05
% Calcium	0.83	0.92
% Phosphorus	0.36	0.39
% Magnesium	0.28	0.31
% Potassium	1.29	1.42
% Sodium	0.409	0.451
PPM Iron	250	275
PPM Zinc	85	93
PPM Copper	15	17
PPM Manganese	70	77
PPM Molybdenum	< 1	< 1

Table 4.5: Nutritional characteristic of the lactation diet

COMPONENTS	AS FED	DM
% Moisture	9.1	
% Dry Matter	90.9	
% Crude Protein	15.3	16.9
% Available Protein	14.9	16.4
% Acid Detergent Insoluble CP	0.5	0.5
% Adjusted Crude Protein	15.3	16.9
% Acid Detergent Fiber	17.8	19.6
% Neutral Detergent Fiber	30.3	33.4
% TDN	65	71
NEL, (mcal/kg)	1.52	1.67
NEM, (mcal/kg)	1.53	1.69
NEG, (mcal/kg)	0.98	1.07
% Calcium	0.77	0.85
% Phosphorus	0.37	0.4
% Magnesium	0.29	0.31
% Potassium	1.23	1.35
% Sodium	0.398	0.438
PPM Iron	231	254
PPM Zinc	81	90
PPM Copper	15	16
PPM Manganese	67	73
PPM Molybdenum	< 1	< 1

Blood Sampling

From 34DIM period until 65 DIM blood samples were taken every 4 days from each cow via coccygeal venipuncture and collected into a vacuum tube (Becton Dickinson Vacutainer System, Franklin Lakes, NJ, USA) containing EDTA (100 U/mL of blood). Each tube was used for hormones and metabolites analyses in plasma. Samples were harvested within 20 min after collection by centrifugation (2,800 x g for 15 min at 4°C) and aliquots of plasma were stored at -20° for analyses as described by Harris et al. (Harris et al., 2007).

Ovarian follicular activity

Ovarian follicular activity of all cows was examined by linear array ultrasonography using a 7.5-MHz transrectal transducer (Ibex Pro; E.I Medical Imaging) on DIM 26, 29 and, from DIM 33 to DIM 65 every other day.

Diameter of follicles between ultrasound examinations was calculated by linear interpolation. Follicles were considered dominant when a diameter of $\geq$ 10mm was reached in the absence of other large, growing, follicles (Savio et al., 1990). An exception to this rule occurred when codominant follicles were observed.

Follicular Aspiration and biopsies

To recover follicular fluid, transvaginal follicular aspiration was performed according to the procedure described by Manik et al. (Manik et al., 2003). Briefly, cows received an injection of xylazine (Anased® 20mg/ml) via coccygeal vessel to induce the analgesia state followed by epidural anesthesia (5cc 2% Lidocaine). A 5.0 MHz transvaginal convex transducer (Ibex Pro; E.I Medical Imaging) fitted to a single lumen aspiration needle (17gx600mm; COVA NEEDLE "type C"; Misawa Medical Industry Co., LTD; Tokyo, Japan) was inserted through the vagina. Follicular aspiration was done on DIM 34, 42, 50. All the follicles with a diameter $\geq$ 10mm were aspirated.

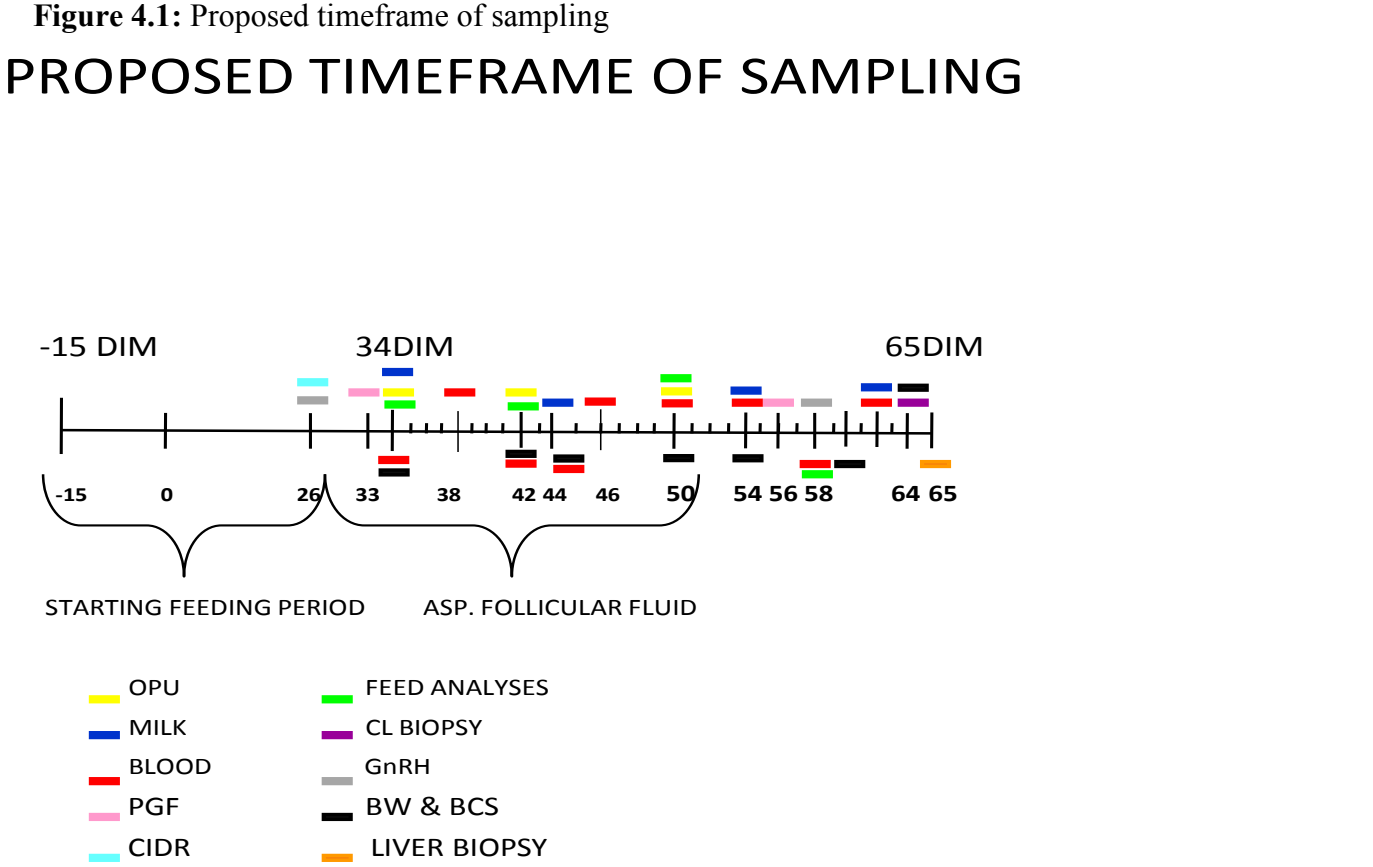
Follicular fluid was centrifuged for 7 min at 3500 rpm at 4°C for granulosa cells and follicular fluid analyses (LDL receptors for granulosa cells; hormones and lipoproteins for follicular fluid). After the centrifugation, the supernatant was stored at -20°C; 1ml of RNA Later (RNA*later*® Soln., Ambion) was than added to the pellet to avoid the degradation of the RNA in the granulose cells. Later, the granulosa cells were stored at 4°C overnight and then, at -20°C until the analyses.

On 56 DIM all the cows received an injection (25 mg) of PGF2α (Lutalyse; Pharmaca and Upjohn) and, after 48h, 100µg of GnRH analogue (Cystoreline, Abbott Laboratories, North Chicago, IL) to induce the ovulation and consequently, the formation of a new corpus luteum. On 64, DIM a sample of the CL was collected by colpotomy. Briefly, transrectal scanning of ovaries for detecting ovulation and measurement of maximal cross-sectional luteal area was performed with an ultrasound scanner (Ibex Pro; E.I Medical Imaging) equipped with 7.5 MHz linear array transducer. The cows received an injection of xylazine (Anased® 20mg/ml) via coccygeal vessel to induce the analgesia state and, after that, cows received an epidural anesthesia (5cc 2% Lidocaine). Also the cows received , via jugular vessel, an injection (50mg) of flunixin meglumine (Banamine injectable Solution, Schering Plough Animal Healt) to prevent inflammation and to induce an analgesic effect post-surgery. The ovary was reached by colpotomy. A sample of CL was cutted and the ovary tissue cauterized soon after the cutting by an electrocautery equipment (3M electrosurgical unit, 3M™ Canada Animal Care) plugged to the surgical scissor. After the biopsy, the luteal tissue was immediately placed in an aluminum pouch, frozen in liquid nitrogen

and stored at –80°C until RNA extraction prior to analysis of IGF-1 receptors. (Rhoads et al., 2008a).

On 65 DIM, a liver sample was collected from each cow. Liver samples were collected by needle biopsy as described by Rhoads et al.,(Rhoads et al., 2008b) with minor modifications. A 40 cm^2 area near the 11th intercostal space on the right side was shaved and disinfected with an iodine scrub solution and 70% ethanol. The cows received an injection of xylazine (Anased® 20mg/ml) via coccygeal vessel to induce the analgesia. Then they received, via jugular vessel, an injection (50mg) of flunixin meglumine (Banamine injectable Solution, Schering Plough Animal Healt) to prevent inflammation and to induce an analgesic effect post-surgery. Local anesthesia (12 mL of 2% lidocaine hydrochloride solution) was administered s.c. to desensitize the incision site and a scalpel blade was used to penetrate the skin. A 1 cm-long incision was made at the center of the intercostal space. Liver tissue was collected with a 12-gauge biopsy needle (15 cm in length with a 20-mm notch) and was placed in an aluminum pouch and frozen in liquid nitrogen immediately after collection. Samples were stored at -80 until RNA extraction. Figure 1 shows the sampling timeframe.

Figure 4.1: Proposed timeframe of sampling



Metabolites and hormones analysis in plasma and follicular fluid

In plasma and follicular fluid was determined the concentration of estradiol by radioimmunoassay (Elrod and Butler, 1993; Beam and Butler, 1997); lipoproteins amount was determined on a Dimension Xpand (Dade Behring, Marburg, Germany) using photometric method

Fatty Acid analysis

Milk:

The extraction and the preparation of methyl esters of milk fatty acids was performerd as described by Bernal-Santos et al.(Bernal-Santos et al., 2003). Fatty acid methyl esters were quantified using a gas chromatograph (Helwett Packard GCD system HP G1800 A, Avondale, PA) equipped with a CP. Sil 88 capillary column (100m x 0.25mm i.d. with 0.2µm film thickness; Varian Instruments, Walnut creek, CA) The oven temperature was set at 70°C for 4min, then ramped to 170°C and maintained for 10 min, with a final increase to 225°C held for 15min. Fatty acid peaks were identified using pure methyl ester standards (Nu-Chek Prep, Elysian, MN). A butter oil reference standard (CRM 164; Commission of the European Community Bureau of References, Brussels, Belgium) was also analyzed periodically to control for column performance and to facilitate the calculation of recoveries and correction factors for individual fatty acids.

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Quantitative real-time PCR analysis in the granulosa cells, Corpus Luteum and liver

Total RNA was prepared with a commercial kit (RNasy Mini Kit, Quiagen, Valencia,Ca, USA). Frozen samples were homogenized in the lysis buffer of the kit (QUIazol Lysis Reagent, Valencia, Ca, USA). The homogenate was processed according to the instructions of the manufacturer. The RNA was checked for intactness by electrophoresis in gel (Agilent RNA 6000 Nano Kit). The mRNA was amplified by quantitative real time-reverse transcriptase PCR assay (qRT-PCR) using a commercial kit (SYBR®Green PCR Master Mix; Applied Biosystem, Foster City, CA, USA). The construction of the primers was based on published cDNA sequence (Chen *et al.*1993, Zhu *et al.* 1993; tab.3). The PCR was performed according to the conditions defined by computing the primers sequences. cDNA (25 ng) was amplified with a program consisting of 95 ◦C for 15 s and 60 ◦C for 40 cycles (ABI PRISM 7000 Sequence Detection System, Applied Biosystems). Dissociation curves were generated at the end of amplification to verify presence of a single product. Sample message abundance was determined relative to a dilution curve of pooled liver cDNA. The housekeeping genes used was the Ribosomial Protein S 9 RNA Quantification and melting curves were analyzed with LightCycler software (Roche Diagnostics mRNA levels were normalized to the 18 S rRNA reference gene and expressed as percentage control values. Table 4.6 shows the primers used to evaluate the expression of GH, IGF-I, LDL.

Table 4.6: Sequences used to evaluate the genes expression in liver, Corpus Luteum and granulose cells

GENE	PRIMER SEQUENCE	REFERENCE
Ribosomal protein S9	F:CTCGACCAAGAGCTGAAG R:CCTCCAGACCTCACGTTTGTTC	Janovick-Guretzky et al. 2007
Total Growth hormone receptor	F:GGTATGGATCTCTGGCAGCTG R:CTCTGACAAGGAAAGCTGGTGTG	Rhoads et al. 2008
IGF-I	F: TTGGTGGATGCTCTCCAGTTC R: GCACTCATCCACGATTCCTGT	Rhoads et al. 2008
LDL receptor	F: ACGAGCTGGGCTGCGTCAAC R: AGGGGCTCGTCCGACCAGTC	NCBI: Ensembl:ENSBTAG00000012314

4.3. Statistical analyses

Individual daily milk production and DMI values were reduced to weekly means before analysis and the yields of fat, protein and lactose were calculated using the weekly mean for milk production. For all analyses, significance was declared at $P < 0.05$ and trends at $P \leq 0.10$. Production variables, metabolites and hormones were evaluated by ANOVA for repeated measures using the PROC MIXED procedure (SAS, 2001). The model included treatment, week or day of treatment, treatment by week or day, BW, BW by treatment, interaction and cow within treatment was a random variable. Abundance of RNA in liver and corpus luteum was analyzed using t-test with Satterthwaite procedure. The abundance of RNA in the granulose cells was evaluated by ANOVA for repeated measures using the PROC MIXED (SAS, 2001). Linear regression analysis was performed using the General Linear Models procedure of the GH receptors as dependent variables and IGF-I receptors

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4.4 Results and discussions

Milk production, as the milk quality in terms of proteins, lactose, milk urea nitrogen and somatic cells, was not affected by supplementation with mixture of CLA (figure 4.2; tab.4.6); the results observed about milk production, contrast with previous studies where have been reported modest increases of about 3 to 10% in milk yield when cows were fed similar amounts of *trans-10, cis-12* (Bernal-Santos et al., 2003; Mackle et al., 2003; De Veth et al., 2006);. Instead, as previously shown (Castaneda-Gutierrez et al., 2007), milk fat yield in the CLA group, decreased progressively over the treatment period and by 60 DIM was significantly decreased compared to the Control group ($P<0.005$; figure 4.3). This fat depression is usually considered as an approach of the CLA supplementation, to reduce negative energy balance in early lactation.

Figure 4.2: Least squares means for milk yield in the Control and CLA Treated groups

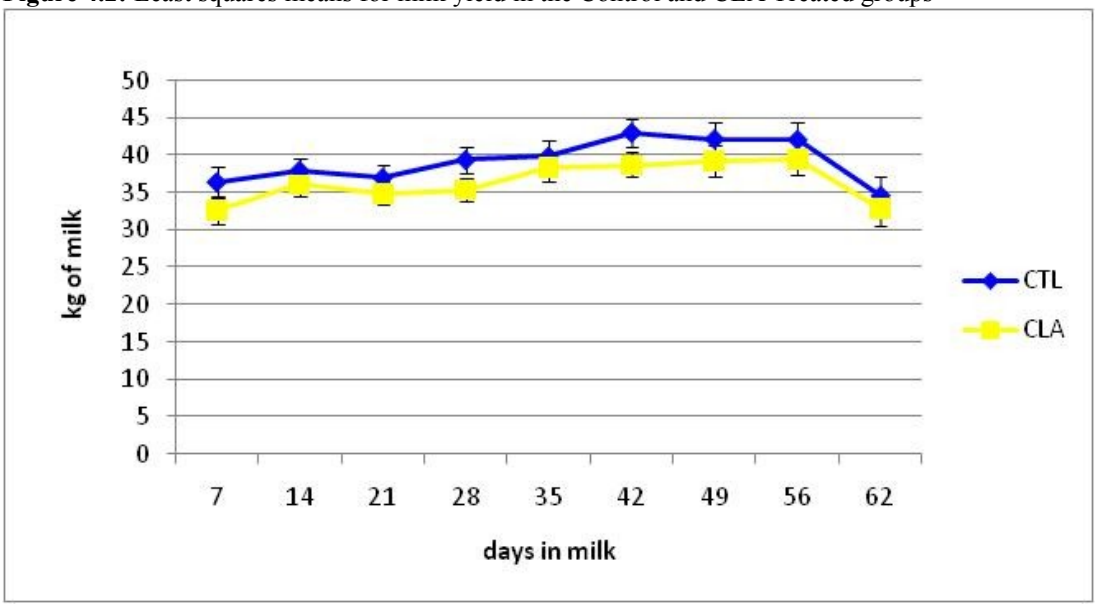


Figure 4.3: Least squares means for milk fat production in the Control and CLA Treated groups

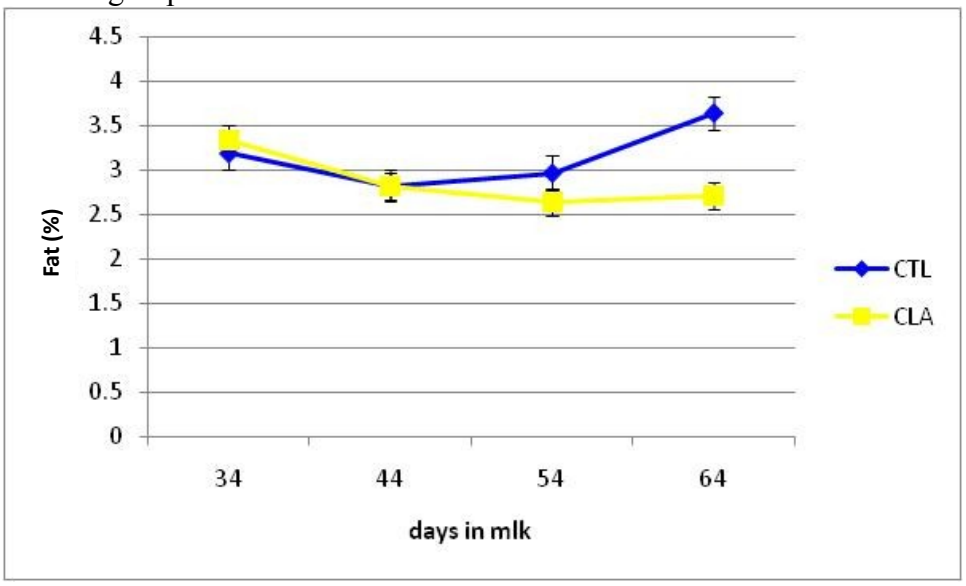


Table 4.6: Milk composition in the Control and CLA Treated groups

	CONTROL	SE	CLA	SE	P
True protein %	2.66	0.002	2.63	0.014	0.684
SCCx1000	356.7	84.42	336.0	71.52	0.913
Mun (mg/dl)	10.14	0.246	9.15	0.209	0.160
Lactose %	5.797	0.789	4.67	0.669	0.299

Fatty acid profile is presented in table 4.7. The CLA cis-9, trans-11 was significantly increased in milk fat of the cows receiving CLA as the trans vaccenic acid (trans11 C18:1), the main precursor of cis-9, trans-11 CLA. Either, no differences were observed about the trans 10 C18:1, fatty acid that usually increase when there is Milk Fat Depression (MFD) Also, no significant changes were observed for the CLA trans-10 cis-12 probably because of its biohydrogenation. Also, the palmitic acid (C18:6) resulted lower in the milk fat of the CLA group.

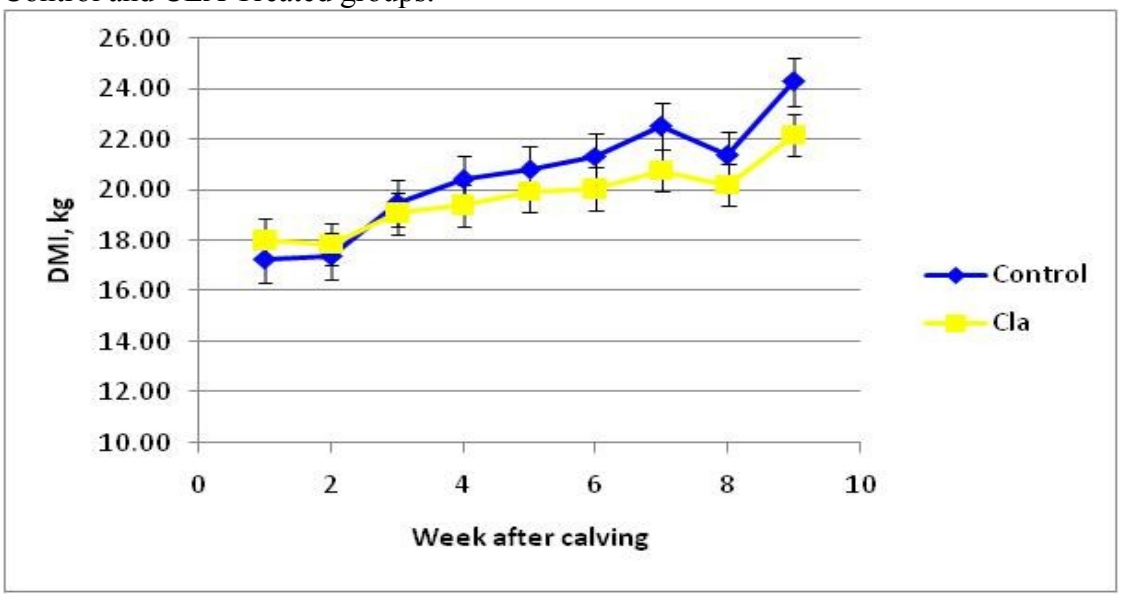
Table4.7: Milk fatty acid composition in the Control and CLA Treated groups

	Treatments				
FA g/100g of FA	**Control**	**S.E.**	**CLA**	**S.E.**	**P**
C4	5.77	0.09	6.08	0.07	0.266
C6:0	2.77	0.07	2.78	0.06	0.932
C8:0	1.26	0.07	1.29	0.06	0.806
C10:0	2.32	0.06	2.19	0.05	0.419
C12:0	2.53	0.05	2.33	0.05	0.262
C14:0	9.44	0.20	9.01	0.17	0.328
C14:1	0.80	0.05	0.73	0.04	0.495
C15:0	0.89	0.05	0.90	0.04	0.937
C16:0	26.68	0.69	25.33	0.57	0.018*
C16:1	1.32	0.05	1.24	0.04	0.424
C17:0	0.64	0.04	0.72	0.04	0.146
C18:0	10.71	0.31	11.26	0.26	0.434
C18:1 t4	0.03	0.06	0.10	0.05	0.392
C18:1 t5	0.02	0.06	0.09	0.05	0.389
C18:1 t6-8	0.45	0.05	0.55	0.05	0.218
C18:1 t9	0.32	0.06	0.42	0.05	0.173
C18:1 t10	1.38	0.09	1.48	0.08	0.793
C18:1 t 11	1.02	0.05	1.31	0.05	0.017*
C18:1 t12	0.65	0.05	0.75	0.04	0.117
C18:1 c9	22.48	0.66	22.44	0.55	0.979
C18:2 c9c12	3.00	0.05	3.3	0.04	0.020*
C20:0	0.10	0.03	0.14	0.03	0.278
C18:3	0.29	0.04	0.35	0.03	0.141
CLA c-9.t11	0.43	0.05	0.56	0.04	0.033*
CLA t-10, c-11	0.01	0.05	0.08	0.04	0.297
Others	2.71	0.82	4.55	0.68	0.216

Regarding the DMI, it was observed that the cows supplemented with CLA had a higher intake during the prepartum period and a less intake postcalving (P<0.01). This particular trend was observed with supplementation of non protected fatty acid because PUFA can cause modifications in the rumen environment and changes in the microbial population that result in decreased fiber digestibility and a reduction in DMI (Palmquist and Jenkins, 1980). This trend was not observed before with rumen protected fatty acids supplementation (Castaneda-Gutiérrez et al., 2005; Castaneda-Gutierrez et al., 2007)

.Our hypothesis is that feed cows with a supplement of fatty acids from the prepartum period might help them to reduce the negative energy balance postpartum. This might be the reason of the decreased DMI after calving observed in the treatment group. However both groups shown a decreased intake after the 8 week after calving that correspond with the time of the ovulation inducted from 56 to 58 DIM (figure 4.4).

Figure 4.6: Least squares means for Dry matter intake (DMI) after calving in the Control and CLA Treated groups.



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From the analyses of Cholesterol, HDL and LDL in plasma, resulted a tendency of LDL to be higher in the group supplemented with CLA, this tendency became statistically significant ($P<0.05$), after 60 DIM In the follicular fluid the same tendency can be observed for the LDL but, in this case, the only significance found was at 32 DIM (fig. 4.7 and 4.8). No differences were observed for Cholesterol and HDL neither in plasma nor follicular fluid (fig.4.9, 4.10, 4.11, 4.12)

Figure4.7: Least squares means of LDL levels in plasma in the Control and CLA Treated groups

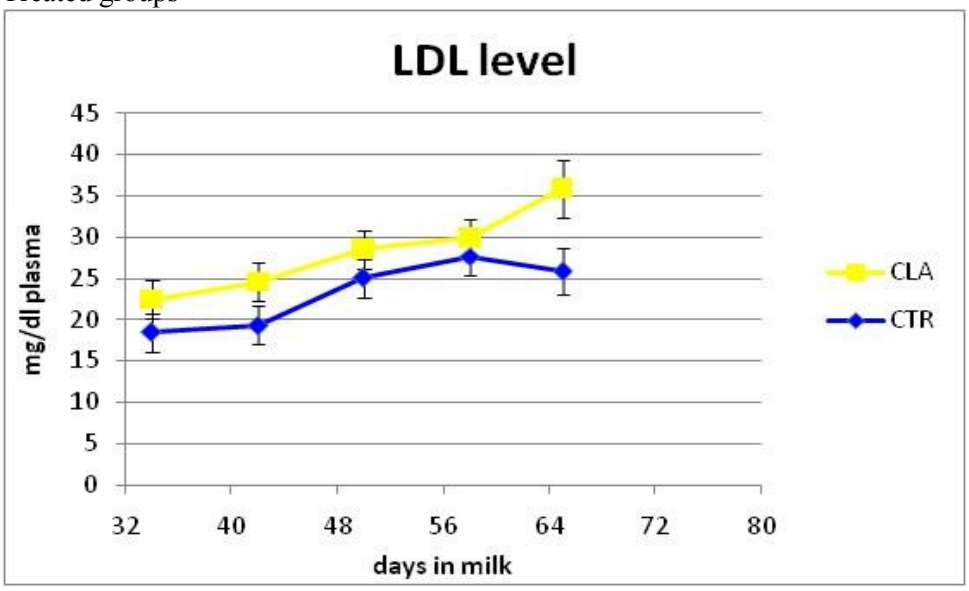


Figure4.8: Least squares means forLDL levels in follicular fluid in the Control and CLA Treated groups

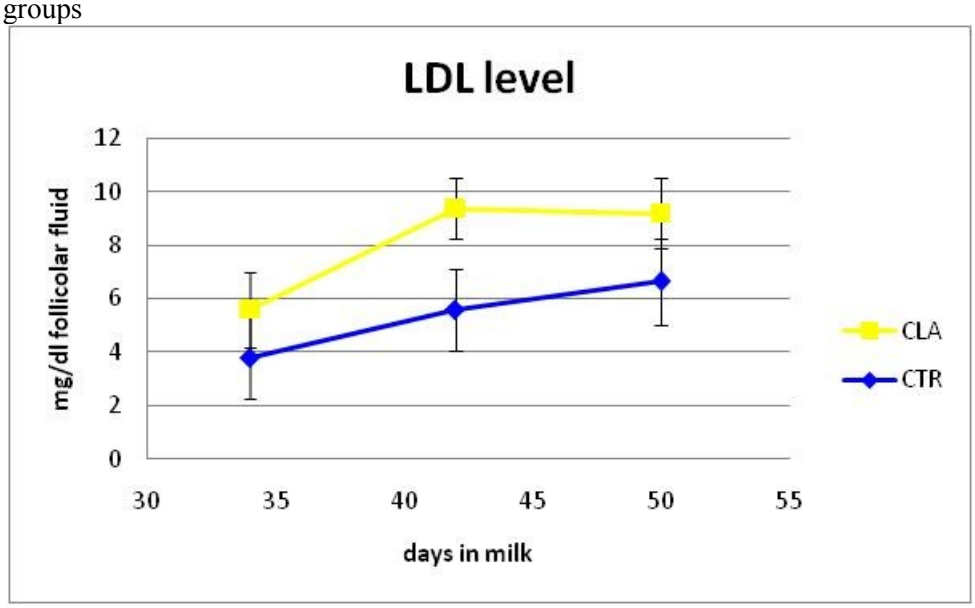


Figure4.9: Least squares means of HDL levels in plasma in the Control and CLA Treated groups

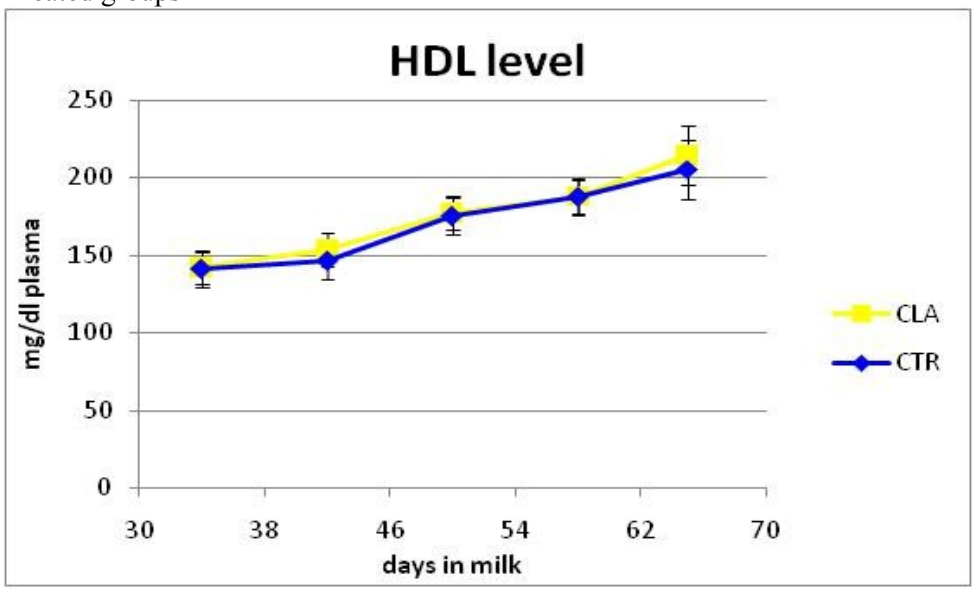


Figure4.10: Least squares means of HDL levels in follicular fluid in the two groups

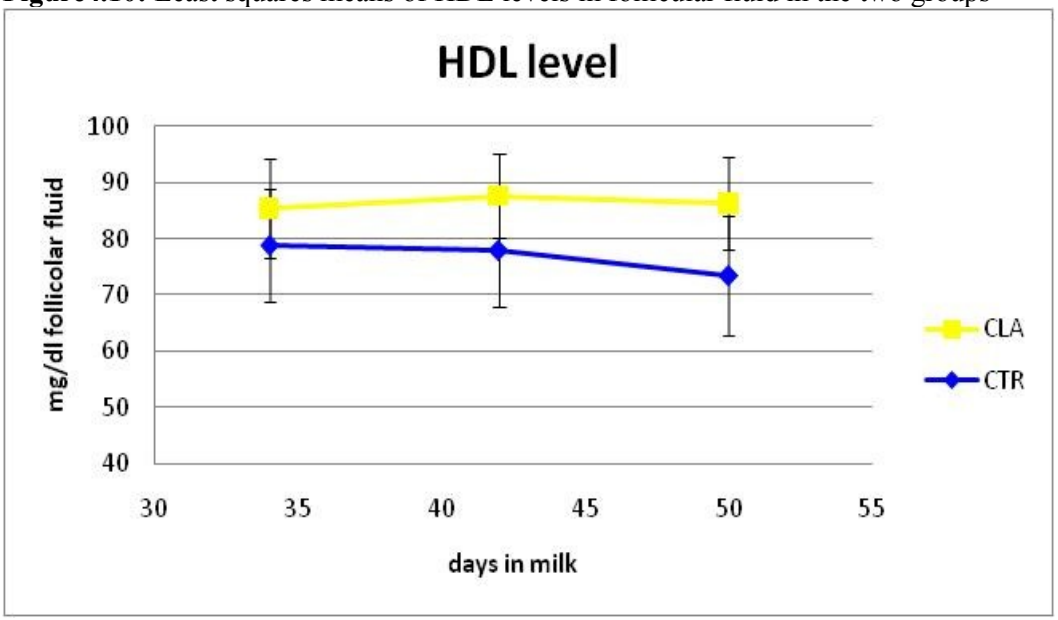


Figure4.11: Least squares means of Cholesterol levels in plasma in the Control and CLA Treated groups

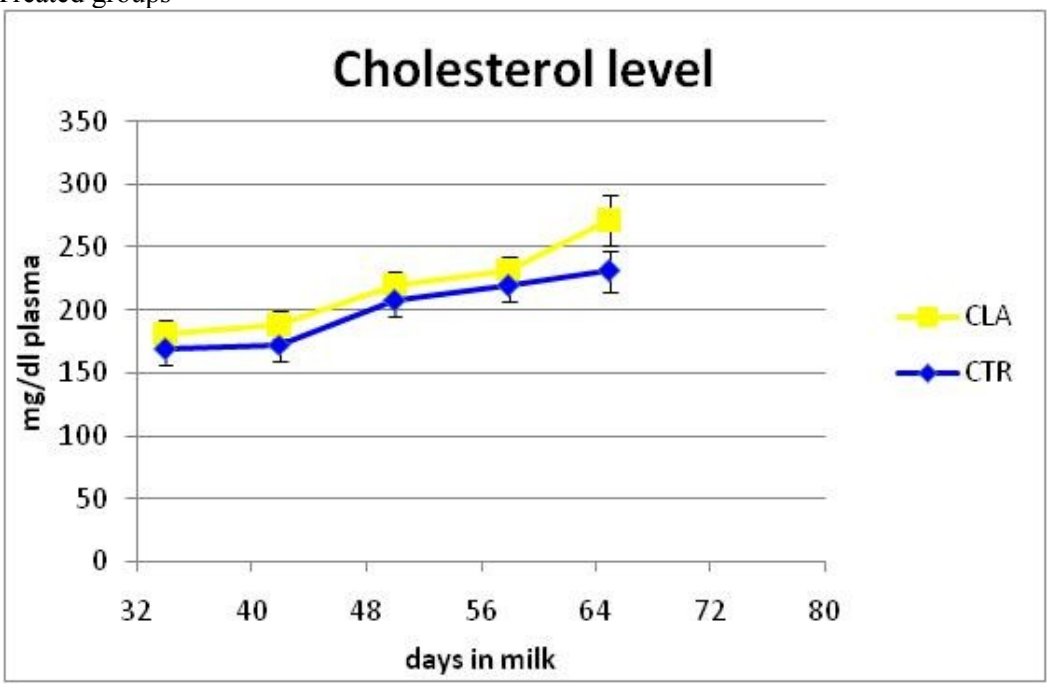
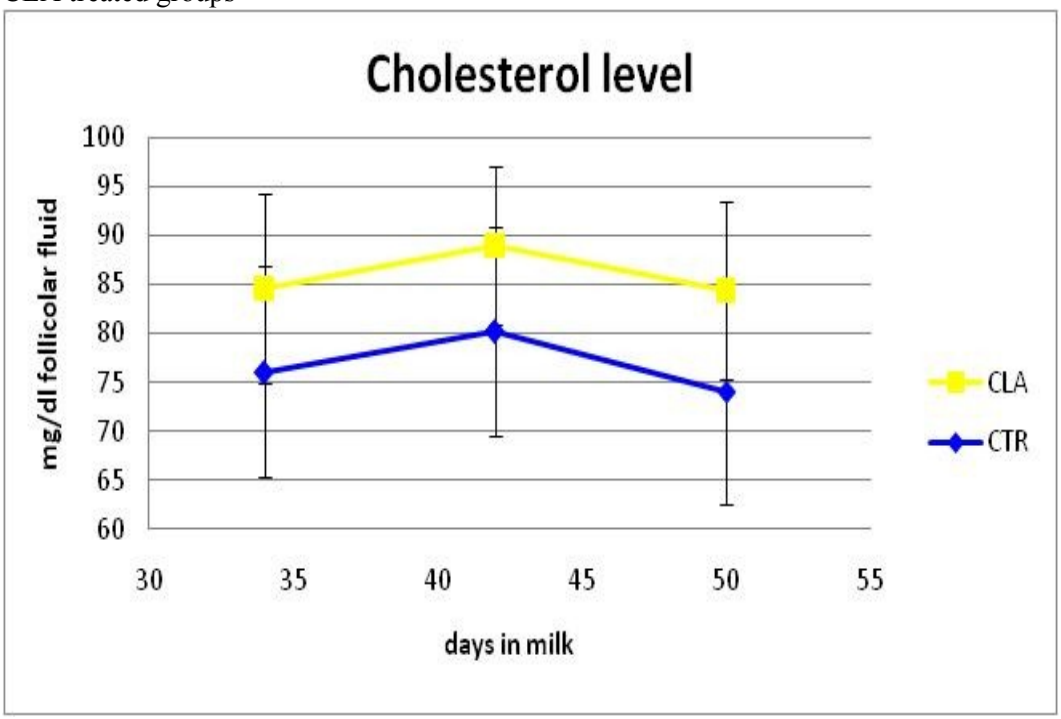


Figure4.12: Least squares means of Cholesterol levels in follicular fluid in the Control and CLA treated groups



Moreover, no differences were observed on plasma levels of estradiol in the two groups. Instead, in the follicular fluid was observed a tendency ($P<0.1$) for higher levels of estradiol, from 42 DIM in the group supplemented with CLA. (figure 4.13 and 4.14). This tendency could explain the trend to higher levels of LDL since several studies observed that levels of LDL are directly correlated with levels of estradiol (Kovanen et al., 1979; Arteaga et al., 1998)

Figure 4.13: Least squares means for levels of Estradiol (E_2) in plasma in the Control and CLA Treated groups

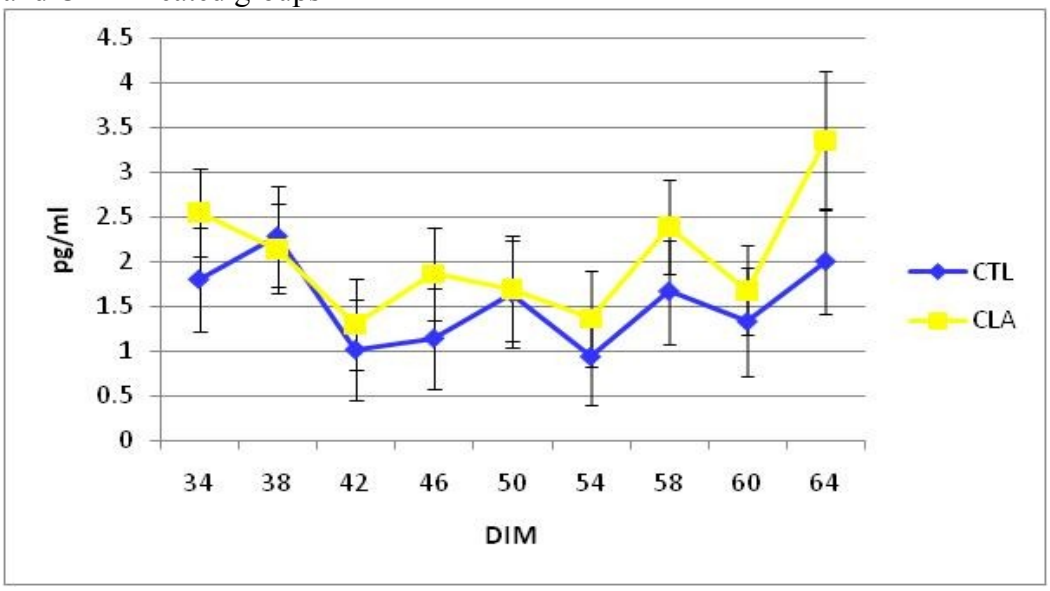
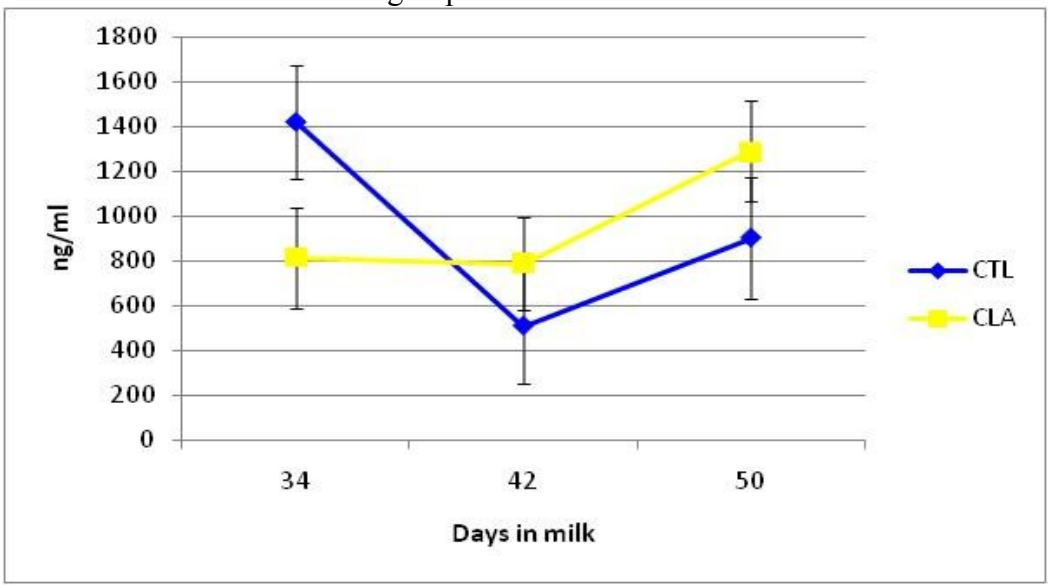


Figure 4.14: Least squares means for levels of Estradiol (E_2) in follicular fluid during in the Control and CLA Treated groups



As said above, the results obtained, in contrast with the literature(de Deckere et al., 1999; Nestel et al., 2006), showed a tendency of higher level of LDL without any change in the level of HDL in the group supplemented with CLA, The reason of this result might be that the amount of CLA offered to the cows was not enough to change the lipoproteins trend. However, the tendency of higher level of LDL could also be explained by the tendency to higher level of estradiol observed(Seeger et al., 1997). Also, since the LDL is used by the granulose cells to produce Progesterone in the luteal phase (Senger, 2003), its higher levels observed in the CLA treated group might be related to higher levels of Progesterone in the luteal phase according to the observation of Castañeda et al. This might reduce the embyo loss during the pregnancy. Castañeda et al., (Castaneda-Gutierrez et al., 2007) found that in cows supplemented with CLA, the progesterone (P4) in the follicular fluid tended to be greater.

Relative mRNA abundance of GH receptor in liver is shown in figure 4.15. Dietary treatment had no significant effect on mRNA abundance, but the assumption of equal variance was violated in the CLA treatment that shows an higher variance of observation. Also, regarding the IGF-I in the liver tissue, no statistical differences were observed beween the two groups (figure 4.16) but, again, the assumption of equal variance was violated in the group supplemented with CLA that also in this case shows an higher variability of the response. The same trend was observed for the evaluation of the IGF-I in the Corpus Luteum tissue (figure 4.17). In the Corpus luteum was also observed a tendency to greater levels of IGF-I in the CLA group. The group supplemented with CLA showed in both the tissues higher variance of the limit of confidence, probably due to the small number of samples evaluated

Figure 4.15: Relative abundance of GH receptor in liver tissue. The CLA group shows an higher variability between the upper (u.c.l.) and the lower (l.c.l) confidence limit compared to the control group

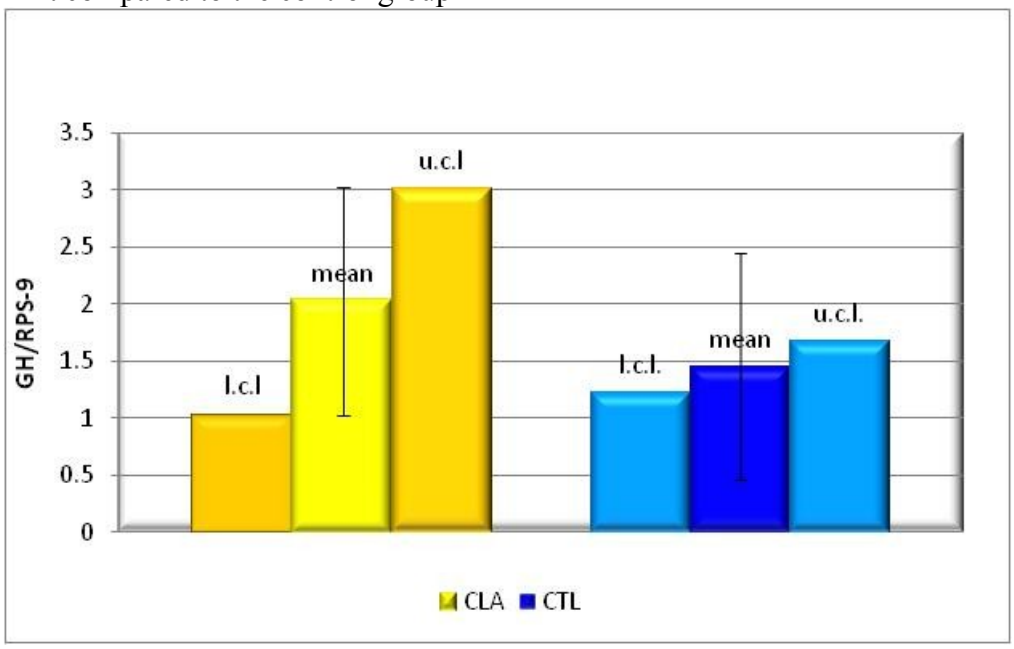


Figure4.16: Relative abundance of IGF-I receptor in liver tissue. The CLA group shows an higher variability between the upper (u.c.l.) and the lower (l.c.l) confidence limit compared to the control group

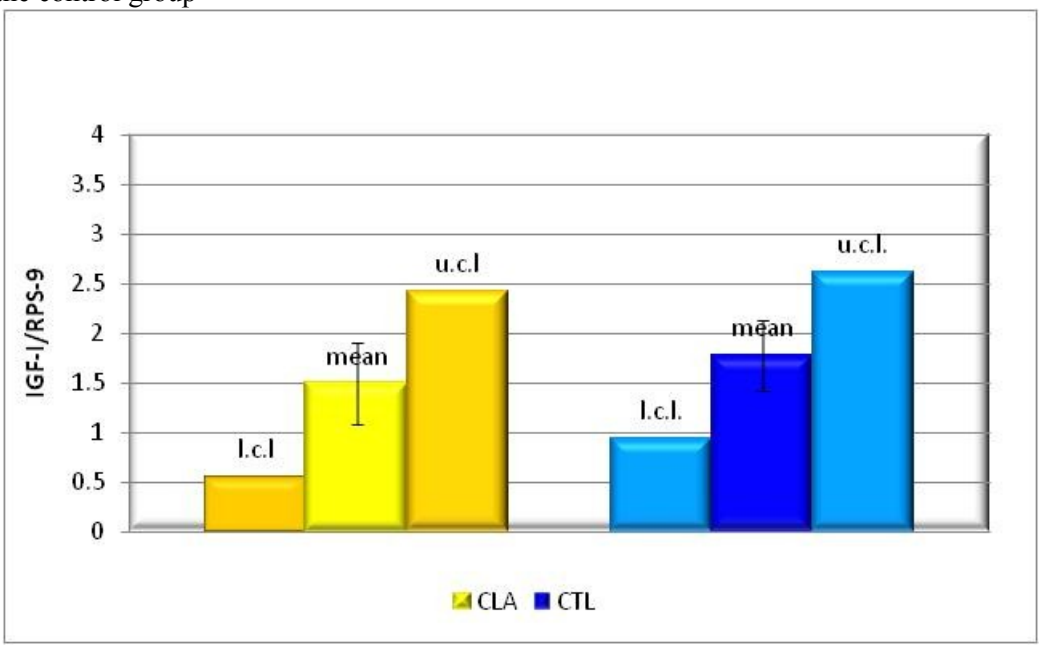
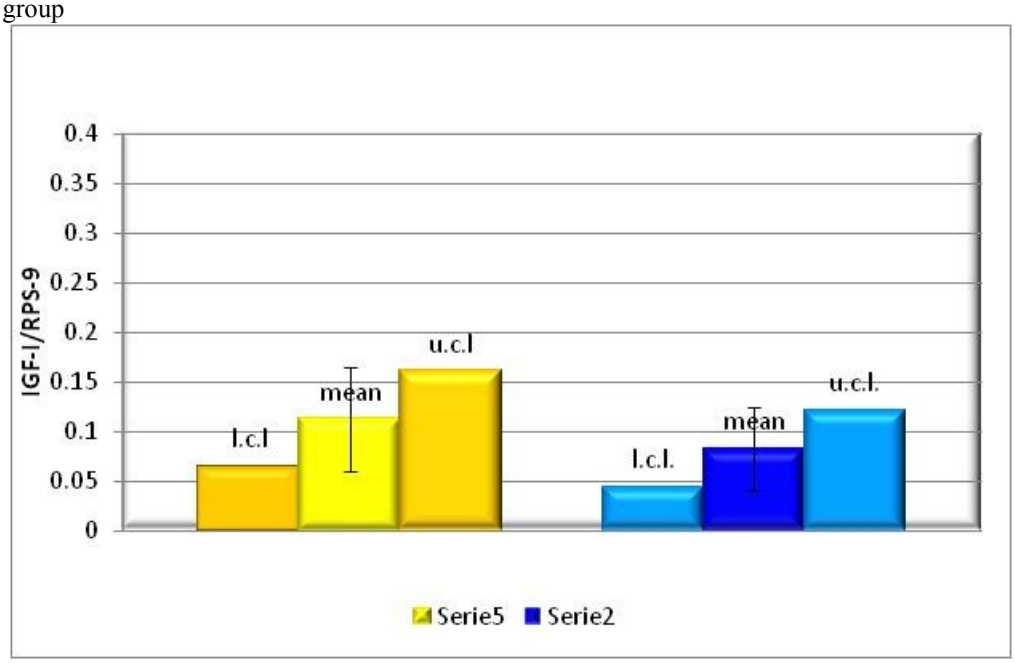


Figure4.17: Relative abundance of IGF-I receptor in Corpus Luteum tissue. The CLA group shows an higher variability between the upper (u.c.l.) and the lower (l.c.l) confidence limit compared to the control group



The analysis of regression between GH and IGF-I receptors in the liver showed, as aspected, a tendency to a positive dipendence of IGF-I for the GH. Anyway no differences were observed between the two groups(fig. 4.18)

The granulose cells were analyzed for LDL receptors after extraction of RNA. Also in thiscase, no difference resulted between the two groups (figure 4.19)

Figure 4.18: Regression between GH and IGF-I receptors in the Control and CLA treated group

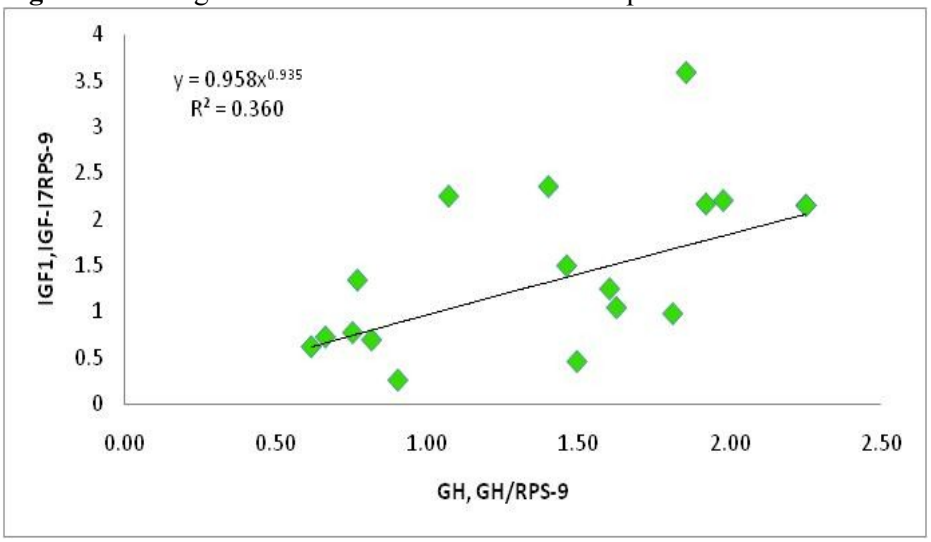
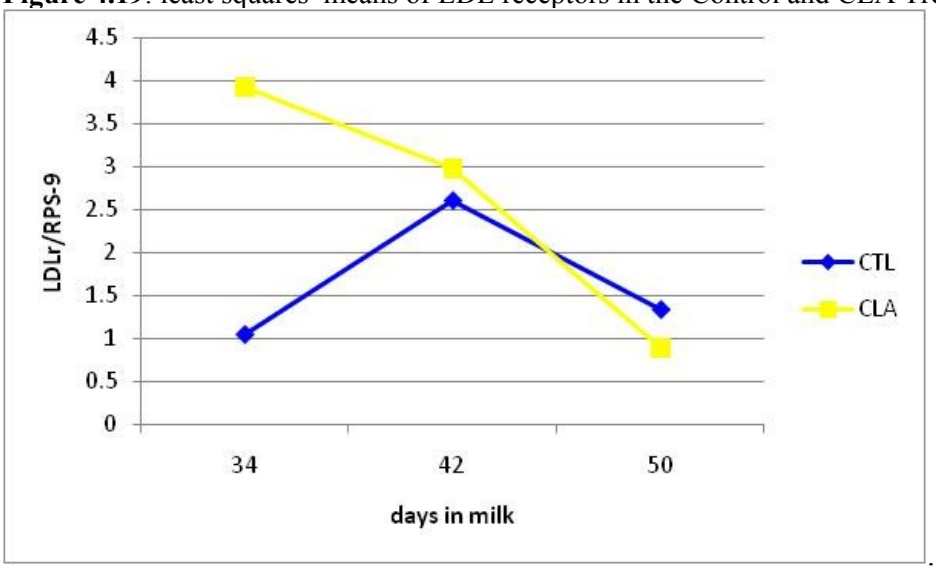


Figure 4.19: least squares means of LDL receptors in the Control and CLA Treated groups



4.5 Conclusions

The present study was conducted to try to explain some of the mechanism of action of Conjugated Linoleic Acid isomers on metabolism and reproduction in dairy cows. Fat supplementation in early lactation may improve reproduction because it can increase energy density of the diet resulting in an improvement in energy balance. In addition, certain unsaturated fatty acids may impact reproduction because they can modify metabolism and gene expression.

Castañeda e tal. (Castaneda-Gutierrez et al., 2007) demonstrated that cows receiving supplement of CLA had higher plasma IGF-I concentrations and tended to have greater progesterone production during the luteal phase and higher ratio of estradiol:progesterone in follicular fluid. The results obtained from this study are not enough to corroborate the data showed from Castañeda et al.because of the small amount of samples observed.

The results obtained from the analysis of RNA, because of the bigger variability of values observed in the samples from cows supplemented with CLA let hypothesize that the response to the CLA supplementation could be mainly affect by subjects. Moreover, studies with larger number of cows are needed to definitively corroborate the effect of CLAs on fertility.

In addition, taking in account the effect of CLA on milk production, consideration in developing nutritional strategies could be useful to establish the optimal daily dose to use and the optimal supplementation program.

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Chapter 5

Conclusions

The aim of this dissertation was to evaluate in dairy cows the effects of the supplementation of Conjugated Linoleic Acid, as natural feed (pasture) or as additive (CLA rumen protected), on quality of dairy products and on cow metabolism and reproduction.

In the first experiment three farms of the Alta Irpina area, (Campania region, Italy) were surveyed focusing on three main features

1. Farm management;
2. Milk yield and quality;
3. Caciocavallo cheese quality

In the farms surveyed, cows' requirements were seldom met due to the poor quality of the forages, the not-rational use and exploiting of natural resources and the inadequate lactating cow management. Essentially the farms in this area need to be supported by technical assistance services that could reduce the production costs and improve the milk productions.

Pasture management and improvement really represent a critical point that may lead negative or positive consequences on the economic vitality of the territory and product quality. Some practical advices for pasture management are related to weed control, rotational grazing, use of fertilizers, and introduction of herbs. However to improve the pasture quality, it's needed to take in account the geological and climatic characteristics of the area and to act by little inputs to avoid loosing of efforts in a not really responsive territory.

The problem highlighted about milk production was, mainly, still related to an incorrect livestock management. As regard milk quality, beside the somatic cells count, it resulted satisfying. The higher count of somatic cells, probably due to the poor hygienic conditions during the milking process, can affect milk yield and quality.

As regard cheese quality, pasture feeding positively influenced the acidic profile of the Caciocavallo cheeses that presented higher level of CLA and PUFA, higher percentage of butyric acid and lower content of palmitic acid. This result represents a straight for Alta Irpinia territory as it could allow to improve the profitability of dairy farms and also it helps to preserve and to enhance the pastures areas.

Feeding factors, in fact, make it possible to vary milk FA composition in many ways. Recent advances in the knowledge of FA synthesis mechanisms (digestion and metabolism) and their putative physiological effects in human consumers have significantly boosted ongoing research and potential applications. As regards ruminant nutrition, the aim, for dairy farmer of Alta Irpinia, should be to better understand the effects and better use grass-based diets

From the sensory test on the caciocavallo cheeses, the only differences noticed were related to the cheese making farm more than to the feeding. These differences also if not really strong, don't allow the characterization of the products.

In conclusion, it can be affirmed that the dairy system of Alta Irpinia area, if technically supported, has a high potential for improvement.

Overall our results constitute objective data for dairy farmer of Alta Irpinia area, as they refer back to the measures to be taken to update or maintain certain feeding conditions (i.e. maintenance of pasture) so that dairy products best reflect the uniqueness and diversity of the native land where they are produced.

In the second experiment the effect of dietary rumen protected CLA on metabolism and reproduction of dairy cows from an intensive farm system in USA was evaluated on the basis of the following remarks.

In the intensive systems, during the last decades, a lot was done to increase milk production. However, this increase in milk output per cow has been accompanied by a worldwide decline in cow fertility. High-yielding dairy cows are typically in a state of negative energy balance postpartum.—Insufficient energy supply results in poor reproductive performance. Fat supplementation in early lactation may improve reproduction because it can increase energy density of the diet resulting in an improvement in energy balance. In addition, certain unsaturated fatty acids may impact reproduction because they can modify metabolism and gene expression.

Considering the results obtained, they indicated that CLA treatment did not affect milk production and quality; only milk fat decreased progressively over the experimental period in the treated cow. As regard milk fatty acid profile, in treated group significant increments were observed for CLA cis-9, trans-11 and trans vaccenic acid, the main precursor of cis-9, trans-11 CLA. No differences were observed for C18:1 trans 10, acid correlated to Milk Fat Depression (MFD) and CLA trans-10 cis-12. Moreover palmitic acid (C18:6) showed lower value in CLA treated group.

Treated cows had an higher intake during the prepartum period and a reduced intake postcalving ($P<0.01$). This particular trend might be due to the fact that the fatty acid supplement from the prepartum period reduced the negative cow energy balance in postpartum period.

Plasmatic and follicular LDL tended to be higher in treated group whereas no differences were observed for Cholesterol and HDL neither in plasma or follicular fluid.

A positive relationship, althought not significant, between GH and IGF receptors was observed in treated cows. Also, the assumption of equal variance was violated in the group supplemented with CLA that shows an higher variability of the response and a numerical tendency to higher values regarding GH and IGF-I receptors This trend, althought not justified statistically could let allow to suppose that by using a larger number of samples an improvement of GH and IGF-I receptor could be shown.

Moreover, in a subsample of cows the glucose level was mesured during the day and the cows supplemented with CLA showed a tendency to lower levels of glucose compared with the control. Since higher levels of IGF-I were reported in cows treated with CLA supplementation, these results might be related to higher levels of insulin and, therefore, to higher levels of IGF-I also in the blood. This hypothesis might allow to hypothesize a direct action of the CLA in the liver.

The level of estradiol tended to be higher in the CLA group as LDL concentration in blood. Since granulose cells use LDL to produce Progesterone in the luteal phase, this result might explain the reason of higher level of progesterone observed in previous studies. Moreover, further analyses are needed to confirm this result.

In conclusion, the data presented in the second experiment suggest that including CLA in the diet can modify endocrine signals that may potentially benefit reproductive outcome. Moreover, studies with larger number of cows are needed to definitively corroborate the effect of CLAs on fertility.

However, milk fat production, from day 60 in milk started to be lower in the group supplemented with CLA. This “negative” effect, also if thought as a way to approach the negative energy balance of cows could lead the farmer to avoid the use of this kind

of supplementation. For this reason, taking in account the effect of CLA on milk production, considering the opportunity to develop nutritional strategies might be useful to establish the optimal daily dose to use and the optimal supplementation program to best use CLA supplementation as improving products quality and fertility.