The influence of native pasture on Conjugated Linoleic Acid and Lipophilic antioxidants content in cows' plasma and milk   I

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Abstract

Summary

CLA and antioxidants are important for the human nutrition. Many human diseases, such as cancer, artherosclerosis and diabetes have been related to low ingestion in the diet. Dairy products represent the nutrients which are richest in CLA.

Milk Antioxidants are less important for human nutrition. However, antioxidants are essential for milk stability because they are able to prevent milk oxidation.

It has been widely reported that a considerable amount of variation in CLA and antioxidant content of milk fat exists. This variation can be attributed to several factors such as: animal diet composition, management systems, animal species, breed, stage of lactation and age of animals.

For example, green forage relative to conserved forage feeding increased CLA and antioxidant in milk. Geographical locations, as well as plant species have been related to the same parameters.

It has also been demonstrated that sheep and goats have less beta carotene in the milk compared to cows. These differences of might be explained, at least in part, by different absorption of beta carotene from the intestine. Other authors have shown, that there are differences between Brown Swiss, Holstein-Friesian, and Jersey breeds with respect to the activity of the mammary enzyme stearoyl Co-A desaturase. This enzyme oxidizes C16:0 and C18:0 to C16:1 and C18:1 and is involved in CLA production. Milk fat content and in consequence, also CLA and antioxidants contents might be related to stage of lactation.

In another study, older cows (>7 lactations) had higher CLA in milk than younger cows (1–3 lactations). Age differences in milk fat CLA content were attributed to differences in desaturase enzyme activities and/or fatty acid metabolism and synthesis between older and younger cattle.

Dairy production plays an important role for Sicilian economics. Bovine farming is mainly located in Palermo and in Ragusa, while caprine farming are situated in Agrigento Messina and Caltanissetta.

In regard of the present research studies, it has been decided to focus on the effects of pasture feeding and animal species on contents of CLA and antioxidants in milk. Two major studies have been performed.

The first study dealt with different levels of pasture intake in the diet.

Total CLA, PUFA and antioxidants in plasma and bovine milk were studied in three groups of cows. One group fed TMR (no pasture); the second group fed TMR supplemented with 30% DM of pasture, and the last group fed TMR supplemented with 70% DM of pasture.

Cattle breed, lactation days and milk production level were similar for all the 3 groups of cows. Blood from the jugular vein and milk samples were collected at the same time during the afternoon milking. Samples were transported to the laboratory and stored at -80°C. The experiment was repeated three times. The effect of pasture in the diet was significant ($P<0.01$) for CLA concentration in plasma.

The second study treated the effects of different animal species and bovine races on alfa and gamma tocopherol content in milk in absence of pasture. Milk samples from different species (buffalo, cow, goat and sheep) have been collected between June and July from five farmhouses located on hyblean highlands of South- eastern Sicily.

In the present investigation the discrepancies found about the levels of α-tocopherol in our milk samples could be explained with interspecies variability and the similarity could be the consequence of a combination of feed characteristics. Although α-tocopherol content was within the range reported in literature, the low concentrations might be explained by poor pasture in α- tocopherol in summer and by vitamin decrease in animal's plasma and in milk in answer to a major heat stress.

However, ewes' milk had higher significantly levels and buffalo's milk lower ($p<0.05$) of α-tocopherol than other milk varieties. We cannot compare our results about γ-tocopherol content in milks because lacking data in literature, although its beneficial roles in human health and in protecting foods. However, in our study differences species-specific have been found with a higher significantly content ($P <0.05$) of γ isomer in goat and buffalo milk compared to other milk varieties.

Abbreviations

AA arachidonic acid

ALA alpha-linolenic acid

BCS Body Condition Score

BW Body Weight

CLA Conjugated linoleic acid

CNCPS Cornell Net Carbohydrate Protein System

CP Crude protein

DHA docosahexaenoic acid.

DM Dry matter

DMI Dry matter intake

EPA eicosapentaenoic acid.

FA Fatty acid

GLA gamma-linolenic acid

LA inoleic acid

MFD Milk fat depression

NDF Neutral detergent fibre

NDF Neutral Fiber detergent

NEL net energy for milk production

NFC non fiber carbohydrate.

NSC Non- structural carbohydrates

PS Soluble protein

PUFA Polyunsaturated fatty acids

RA Rumenic acid

RUP ruminal degradable protein

TMR Total mixed ration

VA Vaccenic acid

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Figure 4.2.A: Content of α-tocopherol in milk from different species. Each sample was analyzed in duplicate and each bar represents the mean ± s.d. of six experiments. Tocopherol concentrations are expressed as $\mu g^{\cdot}g^{-1}$ of fat. Significant differences are shown as * and ** ($P < 0.05$).

Figure 4.2.B: Content of γ-tocopherol in milk from different species. Each sample was analyzed in duplicate and each bar represents the mean ± s.d. of six experiments. Tocopherol concentrations are expressed as $\mu g^{\cdot}g^{-1}$ of fat. Significant differences are shown as * and ** ($P < 0.05$).

Figure 4.3: Tocopherol isomers average composition (%) in milk from different species.

To my husband Mauro
And my son Peppino

Chapter 1
Literature Review

1.1 Milk composition in general

Bovine milk contains the nutrients needed for growth and development of the calf, and is a resource of lipids, proteins, amino acids, vitamins and minerals. It contains immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes and other bioactive peptides.

The lipids in milk are emulsified in globules coated with membranes. The proteins are in colloidal dispersions as micelles. The casein micelles occur as colloidal complexes of protein and salts, primarily calcium (Keenan and Patton, 1995). Lactose and most minerals are in solution. Milk composition has a dynamic nature, and the composition varies with stage of lactation, age, breed, nutrition, energy balance and health status of the udder. Colostrums differ considerably to milk; the most significant difference is the concentration of milk protein that may be about the double in colostrum compared to later in lactation (Ontsouka et al. 2003).

The change in milk composition during the whole lactation period seems to match the changing need of the growing infant, giving different amounts of components important for nutrient supply, specific and non-specific host defence, growth and development. Specific milk proteins are involved in the early development of immune response, whereas others take part in the non-immunological defence (e.g. lactoferrin). Milk contains many different types of fatty acids (Jensen & Newburg 1995). All these components make milk a nutrient rich food item.

1.2 Nutraceutical property of milk

The fact that food items may exhibit health benefits beyond their nutritional value has been recognised since a long time. Many traditional recommendations on food selection have included this view. In more recent years the interest in food with specific health benefits has greatly increased

and stimulated the development of respective products for the food market. At the same time large efforts are made to substantiate health claims by validated experimental methods.

Dairy products have become to play an important role in this context. Several studies done on the milk have helped to deepen the knowledge regarding the nutritional and extranutrional characteristics of milk, showing also that it is considered a multifunctional food product. To its already known healthful proprieties, such as proteins, lipids, vitamins and minerals, (Table 1.1) other important beneficial proprieties were found due to the presence of numerous bio-active molecules from which one can draw benefit through the direct consumption of milk or its derivatives (Guimont et al., 1997).

Milk lipids can undergo auto-oxidation, which may lead to changes in food quality. The mechanisms involved include a complex interplay of pro- and antioxidants consisting both of low-molecular-weight compounds, such as vitamins, and proteins. Several studies have focused on increasing the amount of unsaturated fatty acids (UFA) and, in particular, of conjugated linoleic acids (CLA) in milk and dairy products (AbuGhazaleh et al., 2002; Jones et al., 2005; Collomb et al., 2006) since they are claimed to have beneficial effects on human health and disease prevention (Parodi, P.W., 1997). Dairy milk products are the richest source of CLA that are both accessible and acceptable to most consumers. Potential anti-carcinogenic, anti-atherogenic and body fat reducing effects are attributed to CLA. It was confirmed that the daily intake of CLA that would provide cancer protection, is higher than the CLA that is currently being consumed by the average person.

Table 1.1: Milk composition and percent contribution to the daily dietary reference intakes of some nutrients (A. Haug et al. 2007).

Milk component	Concentration in 1 l whole milk[a]	Percent contribution of 0.5 l whole milk to reference intake[b]	Health effects
Fat	33 g/l		Energy rich
Saturated fatty acids	19 g/l		Increase HDL, small dense LDL, and total cholesterol. Inhibition of bacteria, virus
Oleic acid	8 g/l		Prevent CHD, gives stable membranes
Lauric acid	0,8 g/l		Antiviral and antibacterial
Myrisitc acid	3,0 g/l		Increase LDL and HDL
Palmitic acid	8 g/l		Increase LDL and HDL
Linoleic acid	1,2 g/l		Omega-6 fatty acid
Alpha linolenic	0,75 g/l		Omega-3 fatty acid
Protein	32 g/l	30–40%	Essential amino acids, bioactive proteins, peptides. Enhanced bioavailability
Lactose	53 g/l		Lactosylation products
Calcium	1,1 g/l	40–50%	Bones, teeth, blood pressure, weight control
Magnesium	100 mg/l	12–16%	For elderly, asthma treatment
Zinc	4 mg/l	18–25%	Immune function. Gene expression
Selenium	37 ug/l	30%	Cancer, allergy, CHD
Vitamin E	0,6 mg/l	2 %	Antoixidant
Vitamin A	280 ug/l	15–20%	Vision, cell differentiation
Folate	50 ug/l	6 %	DNA synthesis, cell division, amino acid metabolism
Riboflavin	1,83 mg/l	60–80%	Prevent ariboflavinosis
Vitamin B_{12}	4,4 ug/l	90%	Key role in folate metabolism

[a] data from USDA Food Composition Data.
[b] Dietary reference intake (DRI) for men and women.

The lipid fraction of dairy products has been often treated as a health concern because of the relatively high content of saturated and trans fatty acids, which adversely influence plasma cholesterol. However, studies have shown that whole milk was more effective in protecting against cardiovascular disease than skimmed milk (Steinmetz et al. 1994).

Milk fat naturally contains UFA in the range of 25% to 35% depending upon feeding regimen, season, breed and period of lactation. More than 95% of UFA in milk fat is in form of oleic acid (OA) (21–30% of total fat), linoleic acid (LA) (2–2.5%) and α-linolenic acid (1–1.3%) (Collomb et al. 2000a). The concentration of CLA, which is a mixture of different isomers of linoleic acid, can vary within a broad range. Precht and Molkentin (1999) reported average CLA concentrations in milk fat between 0.45 g/100 g in winter to 1.20 g/100 g in summer. Over 60 years ago, Bank and Hilditch (1931) showed that feeding liberal amounts of highly unsaturated oils to steers over a period of 260 days had no effect on the level of unsaturation of body fat.

Later, Shortland et al. (1957) observed that although the main dietary fat in pasture-fed animals is linolenic acid (C18:3), it is only present in trace amounts in the depot fat of ruminants.

The first evidence of ruminal biohydrogenation of dietary lipids was provided by Reiser (1956), Hartman et al. (1960). It was also established that the process of biohydrogenation in the rumen was incomplete, and that unsaturated fatty acids were saturated by ruminal microoganisms.

The presence of conjugated unsaturated fatty acids in milk was first observed by Booth et al. (1935) who reported that milk fat from cows grazing pasture in the summer showed an increased absorption in the ultraviolet region (230 nm) as compared to milk fat produced by the same cows during the winter months. During those years, it was a common practice for cows to graze during the summer months and receive dry forage in the winter months.

Forty years later, Parodi (1976) isolated cis-9, trans-11 C18:2 (c9, t11 CLA) from milk fat and suggested that fatty acids with conjugated unsaturation are not normally part of a cow's diet, but that they appear in milk as a result of ruminal biohydrogenation of lipids. Ten years later, Ha et

al. (1987) isolated CLA from grilled ground beef and showed that synthetically prepared CLA inhibited the initiation of mouse skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene.

Since then, there have been numerous research studies conducted in an attempt to understand the synthesis of CLA, its mechanisms of action, and its content in natural foods.

1.3   CLA isomers

CLA is a group of unsaturated fatty acid isomers that occur naturally in foods derived from ruminants, and is found in its highest concentration in bovine milk (Chin et al., 1992; Dhiman et al., 1999; Kelsey et al., 2003; Aydin et al., 2005;). CLA isomers have been found using silver ion-high performance liquid chromatography and gas chromatography-electron ionization mass spectrometry (Chin, et al. 1992; Sehat, et al. 1998; Shantha et al. 1994; Fritsche et al. 2000).

Conjugated Linoleic Acid is the collective term used to describe the geometric (cis/cis,cis/trans, trans/cis, trans/trans) and positional (double bond position 7 & 9; 8 & 10; 9 & 11;10 & 12; 11 & 13; 12 & 14) isomers of octadecadienoic acid (C18:2) (Aydin et al., 2005; Rickert et al., 1999; Stanton et al., 1997) (Table 1.2) containing a conjugated unsaturated double bond system, which consists of two double bonds, separated by single carbon – carbon bonds instead of a methylene group (Chin et al., 1992; Dhiman et al., 1999a,b; Parodi., 1999; Dhiman et al., 2000; Donovan et al., 2000; Griinari et al., 2000; Dugan et al., 2001; AbuGhazaleh et al., 2002; Parodi., 2003).

CLA is therefore a category and cis-9, trans-11 C18:2 CLA is a unique molecule in that category. This is important as different isomers have different attributes and some, unlike others, may be beneficial for animal and human health (Kelly et al. 2001).

Table 1.2: The mean positional and geometric isomer composition (% of total isomers) and the CLA content of samples of milk, butter, cheese

CLA isomer	Milk[1]	Butter	Cheese
cis, trans isomers			
7, 9	5.5	6.7	3.6
8, 10	1.5	0.3	1.0
9, 11	72.6	76.5	83.5
10, 12	0.4	1.1	-
11, 13	7.0	0.4	4.7
11, 13	-	-	-
12, 14	0.7	0.8	0.4
Total *cis, trans* (*trans,cis*)	87.7	85.8	93.2
trans,trans isomers			
6, 8	-	0.1	0.7
7, 9	2.4	-	0.6
8, 10	0.4	-	0.3
9, 11	2.0	-	1.5
10, 12	0.6	-	0.5
11, 13	4.2	-	2.3
12, 14	2.8	-	0.9
13, 15	-	-	0.1
Total trans, trans	12.3	9.4	6.3
cis, cis isomers			
8, 10	-	-	<0.1
9, 11	-	-	0.3
10,12	-	-	<0.1
11, 13	-	-	0.3
Total *cis, cis*	-	4.8	0.7
Total CLA (% of fat)		0.5	0.93

[1]Shinqfield et al (2003).

1.4 Health properties of Conjugated Linoleic Acid

The average CLA content of milk in the USA varies between 3 and 6 mg/g of the total fatty acids (Dhiman et al., 2000). The cis-9, trans-11 C18:2 fatty acid, also termed rumenic acid (Kramer et al., 1998; Ellen & Elgersma, 2004; Destaillats et al., 2005) is the most biologically active and also the most abundant natural isomer of C18:2 and accounts for more than 94 % of the CLA in dairy products (Dugan et al., 2001; Collomb et al., 2004; Dhiman et al., 2005).

According to Pariza (1999) there is emerging evidence that rumenic acid (RA) and trans-10, cis-12 C18:2 CLA isomers may be responsible for

different biological effects, and in some cases they may have a cumulative effect. The structures of c9, t11 CLA, t10, c12 CLA, and C18:2 are shown in Figure 1.1.
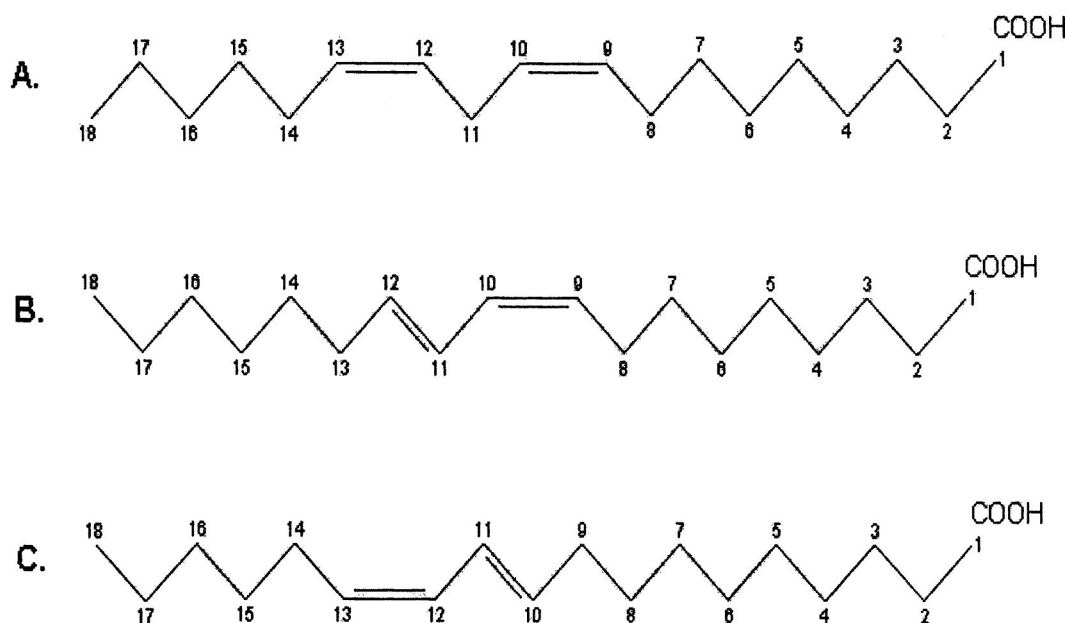
Figure 1.1: Abbreviated chemical structures of ordinary C18:2 (linoleic acid) (A) two major conjugated linoleic acids: *c*9, *t*11 isomer (B) and *t*10, *c*12 isomer (C).

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

Apart from being an effective anticarcinogen, CLA also has numerous properties that could be beneficial to humans and include: antiatherogenic, immunomodulating, growth promoting, and lean body mass-enhancing properties (Parodi, 1999; Cook et al., 2003; Selberg et al., 2004; Weiss et al., 2004a, b; Aydin et al., 2005).

The mechanisms whereby this occurs are not known, but some theories are that CLA reduces cell proliferation, alters various components of the cell

cycle, and induces apoptosis (Belury, 2002). In several human cancer studies, an inverse association was found between the level of CLA in the diet and the risk of developing cancer in breast adipose tissue (Durgam et al. 1997, Thompson et al. 1997, Visonneau et al. 1997, Bougnoux et al. 1999).

Studies conducted with mice, chickens, and pigs have suggested a possible role of CLA (mainly the trans-10, cis-12 isomer) in decreasing body fat and increasing lean body mass (Park, et al. 1997, West et al. 1998). A human-related study has suggested that CLA increases body mass without increasing body fat (Kreider et al. 2002). Several studies indicate that CLA may enhance immune function.

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

The latter approach is more practical since it can be manipulated through the diet of the ruminant. Therefore increasing the CLA content of milk has the potential to increase the nutritive and therapeutic value of milk.

Milk fat CLA is almost entirely rumenic acid (RA), which makes milk the most natural source of CLA, with reported values ranging from 2.4–28.1 mg/g FA (Parodi, 1997). Ip et al. (1994) suggested that an intake of 3.5 g CLA /day for a 70 kg person should be sufficient for cancer prevention. According to Dhiman et al. (2005) whole milk contains on average 3.5 % milk fat of which 0.5 % is CLA. Therefore, one serving (227 ml) of whole milk and one serving of cheese (30 g) can provide 90 mg of CLA. This is only 25 % of the amount suggested by Ip et al. (1994).

However, Knecht et al. (2001) found that the risk of breast cancer was halved in women who consumed more than 620 ml of milk per day, compared to those consuming less than 370 ml a day.

Ritzenthaler et al.(2001) estimated the actual average CLA intake of humans to be 150 mg/d. Again assuming a daily food intake of 600 g, this level of CLA still only amounts to 0.025% of the diet.

For this reason, increasing the CLA contents of milk and meat has the potential to raise the nutritive and therapeutic values of meat and dairy products. The intake of CLA in the human diet can be increased either by increasing the consumption of foods of ruminant origin, or by increasing the CLA content of milk and meat. As the latter approach is more practical, several research studies have been conducted during the last decade in an effort to enhance the CLA content of milk and meat. It is evident from the table 3 below that products from ruminant animals contain the highest concentrations of CLA, of which milk products rank the highest.

Table 1.3: Food sources of CLA. Adapted from Chin et al. (1992)

Food source	Content mg/g fat	% Rumenic acid
Condensed milk	7	82
Milk fat	5.5	92
Buttermilk	5.4	89
Lamb	5.4	92
Processed Cheesed	5	83
Butter	4.7	88
Ice cream	3.6	86
Other dairy products	6.6-7	82
Natural cheeses	2.9-7.1	83
Yogurt	1.7-4.8	82

1.5   Synthesis of CLA

CLA is formed by the rumen gut micro organisms by microbial isomerization of dietary linoleic acid and desaturation of oleic acid derivatives. When polyunsaturated fatty acids (PUFA) in the diet enter the rumen, they are extensively modified through biohydrogenation by rumen micro-organisms with the help of lipases hydrolyzing triglycerides, phospholipids and glycolipids (Khanal & Dhiman, 2004). The CLA is produced by ruminal biohydrogenation or by endogenous synthesis in the tissues (Figure 1.2). In the rumen, CLA is an intermediate in the biohydrogenation of linoleic acid from dietary fat by the rumen bacteria Butyrivibrio fibrisolvens and, in the tissues, CLA is synthesized by Δ9 desaturase from vaccenic acid (trans-11 C18:1) (Ellen & Elgersma, 2004; Destaillats et al., 2005). The endogenous synthesis of CLA from VA has been proposed as being the major pathway of CLA synthesis in lactating cows, accounting for an estimated 78% of the CLA in milk fat (Griinari et al. 2000, Corl et al. 2001).
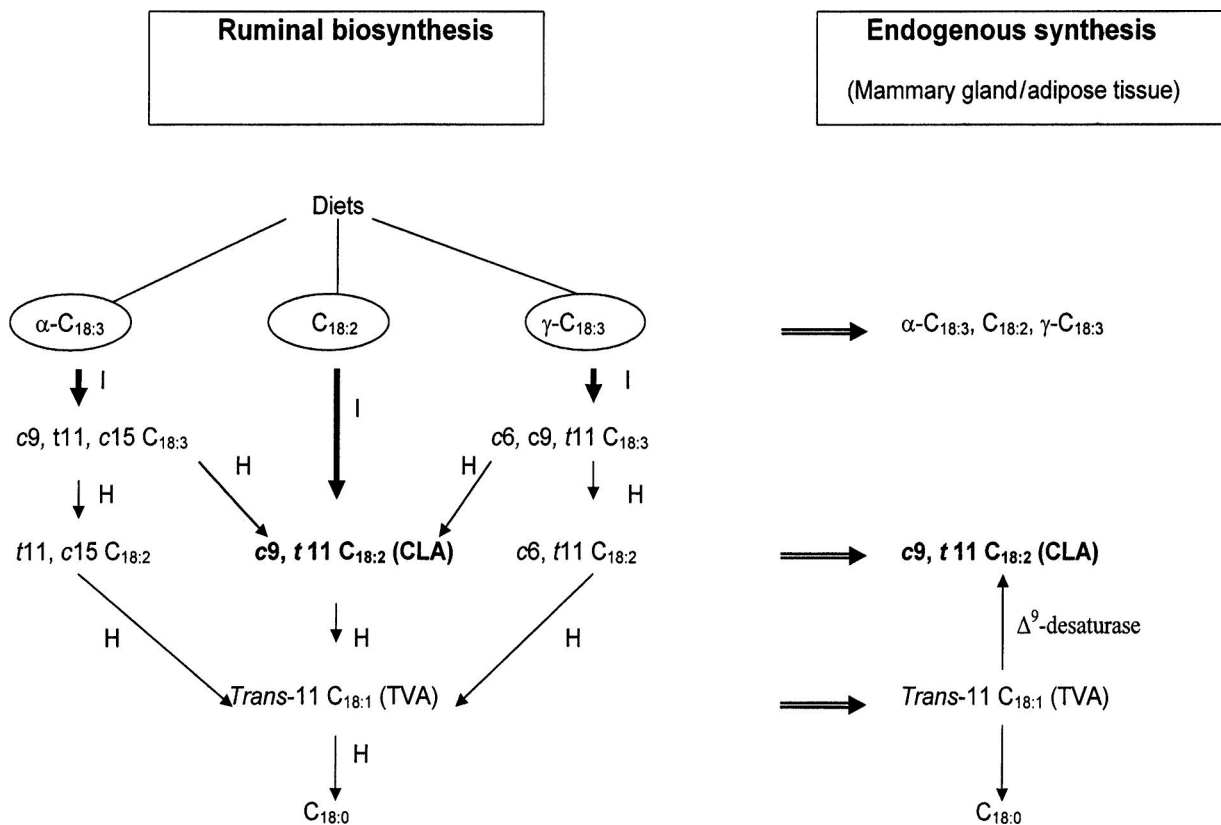
Figure 1.2: Mechanism for CLA synthesis from ruminal biohydrogenation or endogenous synthesis.

When consumed by ruminants, the lipid portions of these feeds undergo two major processes in the rumen (Dawson et al. 1997, Demeyer et al. 1995). In the first process, esterified plant lipids or triglycerides are quickly hydrolyzed to free FA by microbial lipases (Jenkins, 1993). In the second process, the unsaturated free FA are rapidly hydrogenated by microorganisms in the rumen to produce more highly saturated end products.

The c9, t11 isomer of CLA is the first intermediate product in the biohydrogenation of C18:2 by the enzyme linoleate isomerase (Figure 1.2), which is produced by the microorganism Butyrivibrio fibrisolvens (Kepler, & Tove, 1967) and other bacterial species. Part of the c9, t11 CLA is rapidly reduced to VA or C18:0, (Kemp et al. 1984; Kellens et al. 1986) becoming available for absorption in the small intestine. Similar to the biohydrogenation of C18:2, the FA α and γ –C18:3, which are the predominant unsaturated FA in forages, also undergo isomerization and a series of reductions, ending with the formation of C18:0 in the case of complete biohydrogenation (Harfoot, & Hazlewood 1988). The c9, t11 CLA and TVA often escaping complete ruminal biohydrogenation are absorbed from the intestine and incorporated into milk fat (Jiang et al. 1996, Griinari et al. 1999). Studies with pure strains of ruminal bacteria have shown that most bacteria are capable of hydrogenating C18:2 to t-C18:1 and related isomers, but only a few have the ability to reduce C18:2 and C18:1 completely to C18:0 (Fellner et al. 1995). Vaccenic acid (VA) is an intermediate in ruminal biohydrogenation of linoleic (LA) and linolenic acid (LNA) (Abu-Ghazaleh et al., 2002).

By increasing the intake in linoleic or linolenic acid can enhance CLA of milk when dietary oil is accessible to the rumen micro-organisms for biohydrogenation, or to the tissues for endogenous synthesis of CLA (Griinari et al., 2000).

When the dietary supply of unsaturated fatty acid is high, or the biohydrogenation process may be incomplete, VA and CLA can escape the rumen and become available for absorption in the lower digestive tract, thus providing substrate for CLA synthesis in the tissues or CLA for direct absorption (Loor & Herbein, 2003).

Endogenous synthesis is the biochemical pathway responsible for the majority of CLA found in products created from milk fat (Kay et al., 2002; Piperova et al., 2002).

Recently, however, it has been suggested that only a small portion of c9, t11 CLA escapes biohydrogenation in the rumen, and that the major portion of c9, t11 CLA in milk comes from endogenous synthesis in the mammary gland via a pathway involving the desaturation of VA by the Δ_9-desaturase enzyme (Griinari et al. 2000; Corl et al. 2001; Yang, 1999).

Several studies have been performed to confirm that the endogenous synthesis of CLA occurs in the mammary gland by Δ_9-desaturase. Using partially hydrogenated vegetable oil as a source of VA, c9, t11CLAproduction was increased by 17% in milk fat (Corl et al. 2001). In addition, specific inhibitors of Δ_9-desaturase, such as sterculic acid, was infused abomasally into lactating cows to quantify the importance of the desaturase enzyme in CLA production. Inhibition of this enzyme was reflected in the dramatic reduction in the c9, t11 CLA content of milk fat (60–71%). The actual estimated endogenous synthesis of c9, t11 CLA in milk fat was (Griinari et al. 2000; Corl et al. 2001) or 80%, (Lock & Garnsworthy, 2002) of the total c9, t11 CLA, with different correction factors used according to the extent of enzyme inhibition by sterculic oil. There are reported species differences in the tissue distribution, in lactating ruminants, the highest activity of Δ_9-desaturase is found in the mammary tissue (Kinsella, 1972).

Parodi, (1999) established that cis-9, trans-11 C18:2 CLA is the major CLA isomer in milk fat. It was termed rumenic acid (RA), as it is found in such high concentrations in the rumen, and it has been generally assumed that this reflects its escape from complete rumen biohydrogenation.

Although isomerization and reduction reactions proceed in a stepwise fashion, different relative amounts of intermediates and products reach the small intestine for absorption. For linoleic acid, the first two steps of the pathway (biohydrogenation of linoleic acid to rumenic acid) happen more rapidly than hydrogenation of VA so that this intermediate (RA) accumulates in the rumen and is absorbed from the small intestine.

Rumenic acid is produced in the rumen as a stable, first intermediate in the biohydrogenation of dietary LA (cis-9, cis-12 C18:2) by linoleic acid isomerase from the rumen bacteria Butyrivibrio fibrisolvens (Harfoot & Hazelworth., 1988; Stanton et al., 1997; Kim et al., 2000). According to Jahreis et al. (1999), LA may theoretically be transformed into at least 24 isomers containing conjugated double bonds at positions 7 & 9; 8 & 10; 9 & 11; 10 & 12; 11 & 13; 12 & 14. Each of these isomers may exist in the cis/cis, cis/trans, trans/cis or 8 trans/trans configuration. This suggests that several specific isomerases and reductases exist.

Changes in the diet often results in bacterial population shifts that alter the pattern of fermentation and products. Almost all isomers of CLA have been identified in food; however, the most commonly occurring CLA in the diet is RA, which is also biologically the most active CLA isomer.

If the rumen environment is changed so that biohydrogenation is inhibited, for example, by lowering the pH, more of the intermediate will escape the rumen and increase the flow of CLA and VA into the duodenum (Piperova et al., 2000; Qiu et al., 2004a).

1.6   Influence of diet in content of CLA

Factors that may affect CLA production in the rumen include the type and source of dietary carbohydrate that may influence the rates of microbial fermentation in a manner that alters the rate of CLA production or utilization by rumen microbes and ultimately, the concentration of CLA in milk fat. Such an effect could help explain the reported differences in the CLA content of milk fat observed between cows fed fresh forage and cows fed preserved forage.

Sugars such as starch, fructosans, pectins, and soluble fiber content, greatly decline during the fermentation process used to preserve forage. The high concentrations of rapidly fermentable starch, sugars and soluble fiber that are found in immature spring pastures may create a rumen environment and conditions that favor a greater production or a reduced utilization of CLA by rumen bacteria.

Other factors that may affect the rumen environment and microbial population could differ in the grazing animal. For example, passage rate and

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

Several studies have shown that CLA occurs in higher concentrations in milk fat from cows grazing pasture (Parodi, 1997; Palmquist, 2001). The predominant fatty acid in fresh pasture is LNA (C18:3, n-3), and CLA is not an intermediate in its biohydrogenation and therefore the high concentrations found in the milk cannot originate only from the rumen (Harfoot & Hazelwood, 1988; Griinary et al., 2000). It is therefore clear that there has to be another place of synthesis. The intermediate from the ruminal biohydrogenation of LNA is VA (trans-11 C18:1), which according to Palmquist (2001), is the substrate for endogenous synthesis of RA in the tissues.

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

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

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

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

Dietary lipid supplements are often used in formulation of high energy diets for high yielding dairy cows to increase diet energy density, but supplements can also provide substrate for biohydrogenation by rumen bacteria (Chouinard et al., 2001). The content of CLA in milk fat is dependant on ruminal production of both CLA and Trans-11 C18:1 and the tissue activity of $\Delta 9$ desaturase and varies widely among dairy herds and between individual animals. This biohydrogenation in turn is dependent on the supply of substrate in the form of PUFA, and in particular dietary LA and LNA (Dhiman et al., 1999; Jones et al., 2000; Bauman et al., 2001; Kelley et al., 2001).

For maintain a high level of CLA in milk fat involves three components: sufficient dietary supply of C:18 polyunsaturated fatty acids to serve as substrate, maintenance of the biohydrogenation pathway which makes trans-11 C:18 as an intermediate, and inhibition of further hydrogenation of trans-11 C:18 in the biohydrogenation processes. When one of the three components is missing and a substantial enrichment in CLA content of milk fat is not obtained.

Large amount of plant oil supplements fail to increase milk fat CLA if the basal diet is resistant to alterations in rumen biohydrogenation. If the biohydrogenation pathway for formation of trans-11 C:18 cannot be maintained, then enchant milk fat CLA will be transient.

The CLA content in milk fat can be affected by a cow's diet, breed, age, non-nutritive feed additives, such as ionophores, and by the use of synthetic mixtures of CLA supplements. Among these factors, the diet is known to strongly influence the CLA content of milk and includes feedstuffs such as pasture, conserved forages, plant seed oils, cereal grains, marine oils and feeds, and animal fat.

Table 1.4: Factors affecting CLA content in milk

Factors	Effect on CLA	Relevant reference
Diet related		
Pasture related		
Fresh/lush green pasture	Highly positive	Dhiman et al (1999a)
Pasture + full fat extruded soybean	No effect	Khanal et al (2003b)
Pasture + soy oil	No effect	Kay et al (2002)
Pasture + fish oil	Positive	Kay et al (2003)
Maturity of pasture	Negative	Loor et al (2002a)
Diversity in plant species	Positive	Collomb et al (2002b)
Elevation of pasture	Highland>mountain>lowland	Collomb et al (2002a)
Fresh cut pasture	Fresh>conserved	-
High forage diet	Positive	Jiang et al (1996)
High grain diet	Negative	Jiang et al (1996)
Raw oil seeds	Minimal	Dhiman et al (2000)
Roasted oil seeds/meals	Positive	Several
Extruded oil seeds	Positive, better than roasted oil seeds	Several
Plant oils	Positive, better than processed oil seeds	Several
Fish meal	Positive, efficient than plant seeds	Several
Fish oil	Positive, efficient than plant oils	Several
Ca salts of fatty acids	Positive	Chouinard et al (2001)
Marine algae	Positive	Franklin et al (1999)
Rumen pH	> 6.0 pH positive	Martin and Jenkins (2002)
CLA supplementation	Positive	Several
Trans fats	Positive	Porter (2003)
Ionophores	Probably positive	Fellner et al (1999)
Low energy diet	Probably positive	Timmen and Patton (1988)
Animal related		
Species	Ruminants>non-ruminant	Several
Breed	Holstein>Brown Swiss>Normandes>Jersey	Lawless et al (1999)
Stage of lactation	Minimal	Kelsey et al (2003)

There is a substantial variation in milk content of CLA among individual cows consuming the same diet (Kelly, et al. 1998a). Dietary addition of plant oils often results in substantial increases in milk fat concentration of CLA. The form in which plant oils are introduced in the diet has a distinct effect.

In general, addition of free oils increases CLA content in milk fat more than plant oils incorporated in feed materials. Linoleic acids content of the plant oil has sometimes a major determinant of the response, between plant oils rich in linoleic and linolenic acids. Use of the plant oils in ruminant diets is limited because they produced inhibitory effects on rumen microbial growth. A method to minimize this effect is to feed Ca salt of plant oil fatty acids. The slow ruminal release of unsaturated fatty acids from Ca salt also

creates favorable conditions for accumulation of trans C 18:1 fatty acids and a subsequent increase in milk fat content of CLA.

Generally, pasture feeding increases milk fat content of CLA, compared with feeding either a total mixed ration with a similar lipid content or conserved forages, it is apparent that the lipid content of pasture forage and other pasture components altering rumen byhydrogenation produce a synergic effect. The highest reported level of enrichment of CLA in milk fat was produced by a diet with the combination of low forage: concentrate ratio and sufficient level of lipid substrate (Bauman et al. 2000).

Dietary grass, silage and pulp'n brew (mixture of brewers grains/sugar beet pulp) intakes on milk fat CLA concentrations was investigated. Milk fat CLA from cow's receiving silage ad libitum supplemented with pulp'n brew was 1.3 and 1.6- fold higher (P>0.05) than on silage ad lib supplemented with winter grass and silage ad lib, respectively.

Milk from cows on summer pasture yielded 1.8 g/100 g CLA which was 2 or 3.5 fold higher than on ad lib silage alone or supplemented with winter grass or pulp'n is a useful supplement for elevating milk fat CLA content of silage fed cows (Lawless et al. 1998).

Dietary addition of fish oils and feeling high concentrate /low forage diets increase the CLA in milk fat by inhibiting biohydrogenation of trans octadecenoic acids.

The effect of diet (Table 5) on the fatty acid profile of milk fat is substantial and in a study by Lynch et al. (2005), diet was responsible for 95 % of the variance in milk FA. It has been widely researched that CLA content of cows' milk fat can be increased through nutritional and management practices (Kelley et al., 1998a; 2001; Ma et al., 1999).

Supplying additional fat in the diet, using feeds rich in LA and LNA, or grazing cows on pasture have all been shown to increase the CLA content in milk. Linoleic and LNA are of particular importance as ruminal biohydrogenation substrates to produce CLA and trans-11 C18:1.

According to Chouinard et al. (2001), these two FA are present at much lower concentrations in animal fat by-products compared to most plant oils. The type of fat used, and its processing is important, as it can alter the rumen function and therefore influence the pathways of CLA production.

Not only can the addition of fats to the diet of a lactating cow alter the production of CLA, but also more importantly, it can alter the milk yield as well as the milk fat production and influence the DMI of the cow (Palmquist & Beaulieu, 1993; Beaulieu et al., 1995; Dhiman et al., 2000). Even though alterations to the milk fat content are aimed at beneficial components, care also needs to be taken not to increase components that are known to pose a health risk (Offer et al., 1999).

A study by Chouinard et al. (2001) evaluated diets with animal fat supplements in different concentrations. Milk yields were similar; however, a shift in milk fatty acid composition did occur with diets containing the fat supplements. Short and medium chain FA as well as palmitic acid were decreased in a linear manner with increasing dietary levels of tallow and yellow grease. In contrast, substantial increases occurred in C18:1 and to a lesser extent in C18:0. The CLA concentrations of milk fat also increased in a linear manner ($p>0.01$) with increasing dietary supplements of tallow and yellow grease. However, the magnitude of response was small and milk fat concentrations of CLA were relatively low, compared with those observed with dietary supplements from plant oils.

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Fresh herbage is of specific interest as a feedstuff for dairy cows as it is generally a low cost feed and also because of its effects on whole milk composition. Fresh herbage also increases the proportions of C18 FA in milk fat, especially the proportions of RA (Agenäs et al., 2002).

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

by supplementing unsaturated oils (Kelly et al., 1998b; Bessa et al., 2000; Palmquist, 2001).

This is due to the fast growth rate of herbage in the summer months (Banni et al., 1996). According to Parodi (1999), it was documented long ago that the CLA content of milk fat is highest in cows grazing lush pasture, assumed to be caused by the high content of PUFA in fresh forage.

The CLA content increased almost linearly in milk fat of cows that were provided ⅓ and ⅔ or all of the daily feed allowances from pasture (Dhiman et al., 1999; Palmquist, 2001). Grazing animals had 5.7 fold higher CLA concentration in milk than cows fed diets containing preserved forage and grain at 50:50 (22.14 vs. 3.9 mg CLA /g FA). The proportion of C18:3 increased in milk fat as the amount of feed from pasture increased in the diet. Feeding pasture grass in dry form as hay did not influence milk CLA content (Dhiman et al., 1999). Additionally, the endogenous production of CLA in the mammary glands of pasture-fed cows cannot be excluded.

The Δ_9-desaturase activity could differ in the mammary gland of cows grazing on pasture compared to cows fed conserved forages and grains.

Forage maturity and method of preservation also seem to be important factors influencing the CLA content of milk. Cows fed immature forages have higher levels of CLA in milk than cows fed mature forage. Cows fed grass silage cut at early heading, flowering, and second cutting had 1.14, 0.48, and 0.81% CLA in milk fat, respectively (Chouinard et al. 1998). The high C18:3 content of immature grass and its low fiber content compared to mature grass probably interact to increase the production of CLA and VA. Harvesting forage as hay decreases the proportion of C18:3 and total FA in grass, whereas harvesting forage as silage, when carried out properly, does not (Doreau,. & Poncet, 2000). The content of C18:3 FA may decrease when forage is wilted before ensiling, or if there is undesirable fermentation during ensiling (Lough & Anderson, 1973; Dewhurst & King, 1998) The amount of C18:3 FA available to the animal as a substrate for CLA and VA synthesis from fresh grass is much higher than that from hay or silage.

1.7 CLA variation in milk

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

Seasonal variation has a substantial influence (Lock & Garnsworthy, 2003), which can be ascribed to changes in herbage composition on pastures, maturity of grasses, and a change in concentrates being fed. Management systems will also have an effect on CLA content of the herd which can be due to different diets and feeding practices used in different systems (Jahreis et al., 1996; Dhiman et al., 2005). Recent studies suggest that dairy cow breed can also influence the CLA content of milk.

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

Dairy cow management systems also influence the CLA content of milk (Jahreis et al.1997) collected milk samples over a period of one year from three farms with different management systems: 1) conventional farming with indoor feeding using preserved forages; 2) conventional farming with grazing during the summer season; 3) ecological farming with no use of chemical fertilizers to produce forages and grazing during the summer season.

The CLA content was 0.34, 0.61, and 0.80% of fat in milk from cows fed indoors, grazed during summer, and cows grazed in ecological farming conditions, respectively. Reasons for these results could be due, in part, to differences in vegetation or forage quality among the three systems.

Therefore, most of the time, differences in CLA content of milk from cows under different management systems are actually due to the differences in feedstuffs produced under different management styles. An abrupt change in diet of dairy cows from indoor winterfeeding (grass silage, hay, and beets) to pasture grazing sharply increased the level of conjugated dienes in milk fat (Riel, 1963). Depending on the season, CLA content in milk varied from 0.6 to 1.2% of milk fat, with content being higher in spring

and summer than in winter (Riel, R.R. 1963, Jahreis et al. 1999, Mackle et al. 1999, Lock et al. 2003).

These data suggest that the availability of fresh forages in spring and summer increases CLA content in milk fat compared to mature forages in late summer or conserved forages in winter.

There is no difference in conjugated diene content of milk fat between morning and evening milkings (Kuzdzal-Savoie 1961). Elevation above sea level was investigated as a possible factor influencing the CLA content of milk (Collomb et al. 2002). Milk samples were taken from several dairies during the grazing season in the lowlands (600–650 m elevation), mountains (900–1210 m), and the highlands (1275–2120 m) of Switzerland. Milk fat CLA contents were 0.85, 1.58, and 2.34% for the three geographical locations, respectively. Variation in CLA content could be due to differences in plant species and plant fatty acid composition among the three locations.

However, there could also be some unexplained differences in fatty acid synthesis or activity of the desaturase enzyme in cows grazing at the three elevations.

The first factor that would cause variation in individual animals is the population of microorganisms in the rumen of the animal. There are several factors that effect this population, as the micro-organisms are very sensitive to rumen pH, lipid substrates in the diet, forage to grain ratio (Qiu et al., 2004a), passage rate and fluid dilution. Meals size, feeding frequency, bite size and time spent ruminating also differs between cows. Another factor that would cause a variation among individual animals is gene expression and activity of the Δ9desaturase enzyme (Kelly et al., 1998; Bauman et al., 2001; Palmquist, 2001). A difference in CLA content of milk fat has also been reported from cows in different stages of lactation with cows with more than 7 lactations having more CLA in milk fat than cows having 1-3 lactations (Palmquist & Beaulieu, 1993; Dhiman et al., 2005).

Montbeliard cows displayed a tendency to have higher CLA in milk fat (1.85%) compared to Holstein-Friesian (1.66%) or Normande cows (1.64%) grazing on pasture (Lawless, 1999), Holstein-Friesian cows had higher CLA content in milk compared to Jerseys fed diets containing conserved forages and grains (Morales et al. 2000, Capps et al. 1999, Dhiman et al. 2002).

Conjugated linoleic acid content was also higher in milk fat from Holstein-Friesians (0.57%) than for Jersey cows (0.46%) when grazed on pasture (White et al. 2002) , Brown Swiss cows had higher CLA content in milk fat than Holstein-Friesian when fed similar diets (Whitlock, et al. 2002; Capps et al. 1999, Dhiman et al. 2002).

However, Kelsey et al.(2002) found that Holstein-Friesian and Brown Swiss cows fed diets containing conserved forages and grain produced milk fat with similar CLA (0.44% and 0.41% of fat, respectively) (Table 1.5). Ayrshire cows had higher CLA content in milk fat (0.68% of fat) compared to Guernsey and Jersey cows (0.34% of fat) when fed conserved forages at 34% and a grain mixture at 66% of dietary DM (Dhiman et al. 2002).

The average difference in CLA content of milk fat among Brown Swiss, Holstein-Friesian, and Jersey breeds is 15 to 20% when fed similar diets. Brown Swiss cows have inherently higher CLA in milk fat, followed by the Holstein-Friesian and Jersey breeds.

Preliminary work by Medrano et al.(1999) shows that there are differences between Brown Swiss, Holstein-Friesian, and Jersey breeds with respect to the activity of the mammary enzyme stearoyl Co-A desaturase.

This information is important, because stearoyl Co-A desaturase oxidizes C16:0 and C18:0 to C16:1 and C18:1 and is involved in CLA production. Beaulieu and Palmquist (1995) and White et al. (2002) reported that Jersey cows produced 15 and 13% less C18:1 than Holstein cows fed similar diets, respectively, confirming the observation of Medrano et al., (1999) that mammary desaturase activity differs among breeds.

Further understanding of the activity of the desaturase enzyme may offer an explanation as to why there are breed differences in milk fatty acid composition, including CLA.

Table 1.5: The CLA content (% of fat) in dairy products from ruminants

Products	Breed/Species	Diet	Content	Reference
Milk	Holstein	TMR	0.44	Kelsey et al (2003)
Milk	Holstein	All pasture	2.5	Khanal et al (2003a)
Milk	Holstein	All pasture	1.7	Khanal et al (2002)
Milk	Holstein	Pasture + extruded soybean	1.7	Khanal et al (2002)
Milk	Holstein	Pasture + extruded rapeseed	2.5	Lawless et al (1998)
Milk	Holstein	TMR + canola seed	1.4	Ward et al (2002)
Milk	Holstein	TMR + flax seed	1.2	Ward et al (2002)
Milk	Holstein	Pasture + grain mix	0.72	White et al (2001)
Milk	Holstein	TMR + 1% Fish oil	0.73	AbuGhazaleh et al (2003)
Milk	Holstein	Pasture + 150 g Fish oil	3.3	Kay et al (2003)
Milk	Holstein	TMR + 3.6% soy oil	2.1	Dhiman et al (2000)
Milk	Holstein	TMR + 5.3% linseed oil	1.67	Kelly et al (1998a)
Milk	Holstein	TMR + 5.3% sunflower oil	2.44	Kelly et al (1998a)
Milk	Jersey	TMR	0.32	White et al (2001)
Milk	Jersey	Pasture + 5.5 kg concentrate	0.59	White et al (2001)
Milk	Brown Swiss	TMR	0.41	Kelsey et al (2003)
Milk	Normande	All pasture	1.7	Lawless et al (1998)
Milk	Water buffalo	-	0.84	Lal and Narayanan (1984)
Milk	Goat	Various	0.58-1.1	Parodi (2003)
Milk	Sheep	Various	1.2-3.0	Parodi (2003)
Milk	Human	-	0.09-0.49	Park et al (1999)
Cheese	Holstein	All pasture	1.5	Khanal et al (2003a)
Cheese	Holstein	Pasture + extruded soybean	1.4	Khanal et al (2002)
Cheese	Holstein	TMR	0.34	Dhiman et al (1999b)
Cheese	Holstein	TMR + extruded soybean	0.73	Dhiman et al (1999b)
Cheese	Holstein	TMR + extruded cottonseed	0.60	Dhiman et al (1999b)
Cheese	Sheep	-	0.8-2.0	Prandini et al (2001)
Cheese	Goat	-	0.27-0.69	Wolff (1995)
Cheese	Mozzarella	-	0.43	Lin et al (19995)
Cheese	Cheddar	-	0.40-0.47	Lin et al (19995)
Cheese	Swiss	-	0.55	Lin et al (19995)
Yogurt	-	-	0.44	Ma et al (1999)
Yogurt	-	-	0.38	Lin et al (19995)
Butter	-	-	0.61	Chin et al (1993)
Butter	-	-	0.47	Ma et al (1999)
Ghee	Buffalo	TMR	0.50	Aneja and Murti (1990)
Ghee	Cattle	-	0.60	Aneja and Murti (1990)
Sour cream	Cattle	-	0.41	Lin et al (19995)
Buttermilk	Cattle	-	0.47	Lin et al (19995)
Evaporated milk	Cattle	-	0.34-0.64	Lin et al (19995)

Existing data on the relationship between the age of the cow (lactation number) and CLA content in milk fat show variable results. When cows were fed grass-based diets, cows in the fifth lactation or higher had more CLA content in milk (0.59% of fat; $p < .06$) than cows in lactations 2 to 4 (0.41% of fat) (Stanton et al. 1997). However, when cows were fed diets

containing full-fat rapeseed, there was no indication of a relationship between lactation number and CLA content in milk fat (Stanton et al. 1997).

In another study, older cows (>7 lactations) had higher CLA in milk than younger cows (1–3 lactations) (Lal, & Narayanan, 1984). Age differences in milk fat CLA content could be due to differences in desaturase enzyme activities and/or fatty acid metabolism and synthesis between older and younger cattle. Further research is needed to understand the mechanisms involved in differences in CLA production with age of the cow. The CLA content in milk varies from cow to cow, even when the same diet is fed. Jiang et al.(1996) and Stanton et al.(1997) found substantial variation in the CLA content of milk (0.15% to 1.77% of fat) among individual cows fed the same diet. Kelly et al.(1998), observed a three-fold variation in CLA content of milk among individual cows fed the same diet at a similar stage of lactation and producing milk with similar fat content.

These differences could be due simply to differences in desaturase enzyme activities in the mammary gland, age of animals, disease conditions, differences in ruminal metabolism, or other unknown factors.

1.8   Milk fat depression

Milk fat depression (MFD) is a well known occurrence, especially where diets are being supplemented with fats. With the supplementation of diets with CLA, a definite relationship was found between MFD and CLA (Griinari & Bauman, 1999; Baumgard et al., 2000; Bauman et al., 2001). Scientists could establish that MFD was related to an increase in not just trans C 18:1, which would include VA, but specifically to an increase in trans-10 C 18:1 (Griinari et al., 1998).

The trans-10, cis–12 C18:2 is an intermediate in the formation of trans-10 C 18:1 from the biohydrogenation of linoleic and linolenic acid. It was shown by Griinari et al. (1999a) that there is a linear relationship between trans-10, cis-12 C18:2 CLA and trans-10 C18:1. They also identified a curvilinear relationship between the increases in milk fat content of trans-10, cis-12 C18:2 CLA and the reduction of milk fat yield in cows fed different MFD diets.

This was confirmed by Baumgard et al. (2001) and Palmquist (2001) when they established that trans-10, cis-12 C18:2 CLA and not RA is responsible for MFD. Perfield et al. (2004) confirmed that trans-10, cis-12 C 18:2 is responsible for MFD and that trans-8, cis-10 C18:2; cis-11, trans-13 C18:2; and cis-9, trans-11 C18:2 had no effect on milk fat percentage.

Chapter 2
Literature Review

2.1   Antioxidants in milk

There is a growing interest in natural nutrients and non-nutrients that are present in foods that may have health benefits for humans like vitamins and antioxidant.

Milk contains many minerals, vitamins and antioxidants. The antioxidants have a role in prevention of oxidation of the milk, and they may also have protective effects in the milk-producing cell, and for the mammary gland.

Some compounds that have antioxidant properties that are present in milk are synthesized by the cow and other compounds are transferred into milk from the diet.

The antioxidants synthesized by the cow are squalene, cholesterol, riboflavin, lactoferrin, caseins (Cervato, et al. 1999), cysteine, ubiquinone (Page, et al. 1959).

The antioxidant taken up from the diet are vitamin C, carotenoids, vitamin A, lipoic acid (Bingham, et al. 1967), vitamin E, polyphenol or degradation products of polyphenol (Buchin, et al. 2002), selenium, lutein. gossypol from cotton seed (Parodi, 1996).

Carotenoids are involved in the nutritional and sensory characteristics of dairy products, and are potential biomarkers for traceability of cows' feeding management.

Antioxidant compounds that are divided in two groups: fat soluble (vitamin E, carotenoids, β-carotene, α-tocopherol, vitamin D, Vitamin A, tocopherols, lutein) and water soluble (lipoic acid, ascorbic acid, selenium).

2.2   Antioxidants from diet to milk

The fat soluble and water soluble antioxidant are transferred from diet to milk in the cow which two different pathway. The transfer of water soluble

antioxidant compounds from the diet, for example Vitamin C, is by active transport in the small intestine. There is an ouabain-sensitive sodium dependent saturable active transport system for ascorbic acid in the brush border of the duodenum and upper ileum, and another sodium-independent transfer process in the basolateral membrane (Baste, et al. 1994). Dehydroascorbic-bate is absorbed by a carrier-mediated passive mechanism, both in the intestinal and in the buccal mucosa. At low levels of intake, vitamin C is very efficiently absorbed and retained by humans (Oste et al. 1997).

The transfer of fat soluble compounds, like carotene, from the diet to milk is facilitated by the interaction with bile in the digestive system and higher levels of fat in the diet. The fat soluble antoxidants are taken up at the intestinal mucosa and incorporated in chylomicra. The chylomicra are transferred through lymphatic system and then are transferred to the blood stream. The lipid material is processed at the liver and then is carried to the mammary tissue by LDL fraction. The uptake of lipid materials at the mammary cell will probably be in conjunction with uptake of other neutral lipids.

Recent studies have demonstrated that milk produced from animals fed on fresh pasture contains higher levels of natural antioxidants (carotenoids and vitamin E), than milk obtained from animals fed with intensive feeding systems (Pizzoferrato & Manzi, 1996). In fact, the transformation and conservation of fresh forage from the pasture for use in the preparation of hay and silage, causes degradation and inactivation of many of these moleculars at different levels (Bruhn and Oliver, 1978; Parker et al., 1983; Thafvelin and Oksanen, 1966).

2.2.1   Absorption of antioxidants

For milk, both the low molecular weight water soluble antioxidants (e.g., Vitamin C) and fat soluble antioxidant compounds (carotenoids) can be absorbed from the diet by humans.

There is very little specific data from human studies, there is some in animal models.

In general, the data for humans are from epidemiological studies and indicate a positive correlation of diets high in fruits and vegetables with lower incidence of cancer, with the assumption that the reduced incidence of cancer is related to antioxidant properties of the compounds in fruits and vegetables. However, even work in animal models on crude preparations of antioxidants from plants has failed to relate the effect specifically to individual compounds or specific mechanisms.

Carotenoids are clearly a class of compounds that can be transferred from feed to milk in the dairy cow and these are retained in the cheese during cheese making.

The ability of the lipid soluble carotenoids to quench singlet molecular oxygen (Martin et al., 1999) may explain some anticancer properties of the carotenoids. β-carotene influences carcinogenesis through a number of mechanisms associated with its in vivo conversion to vitamin A. Mediated by nuclear retinoic acid receptor, vitamin A regulates the expression of genes that regulate cell growth and differentiation (Parodi, 1999).

Carotenoids are absorbed intact by a nonsaturable passive mechanism, that is not mediated by a carrier. Dietary vitamin A in the form of esters is hydrolysed during digestion and is absorbed in the free form from proximal small intestine. Bile salts, pancreatic lipase and fat aid in the absorption of both vitamin A and β-carotene (Parodi, 1996). In the intestine the absorption is facilitated by the formation of bile acid micelles.

The hydrocarbon structure of the carotenoid prevent them from being water soluble and, like other non polar lipids, are solubilized within the gastrointestinal tract when micelles are formed. Micellar solubilization facilitates the diffusion of lipids across the unstirred water layer (Hollard, et al. 1981). Beta-carotene can either be absorbed intact or cleaved at the central double bond in the intestinal mucosa to form two molecules of retinal which is to reduce to retinol.

Retinol generated from β-carotene as well as that absorbed directly is esterified, mainly with palmitic acid within the intestinal mucosa. Incorporated into chylomicrons, the retinyl esters pass via lymph to the general circulation and are carried to the liver for storage and subsequent distribution (Parodi, 1996). Then the carotenoids are incorporeted in the

LDL or HDL, so this lipoprotein is transferred through the blood stream to the capillary bed of the mammary cells. The lipids are released from the lipoprotein carrier and are absorbed across the mammary epithelium into secretory cells. In milk, carotenoids are found inside the lipid-carrying milk-fat globules and are not specifically associated with the MFGM (Patton, et al. 1980).

β-carotene is an antioxidant and has free radical quenching properties thus protecting against oxidative DNA damage. While retinoids protect against the early stages of carcinogenesis, β-carotene protects against cancer progression.

And also some anticarcinogenenic effects of vitamin A and β-carotene may be mediated through their immune-stimulating effect (Parodi, 1996).

Vitamin E is absorbed in the same path as other non polar lipids such as triglycerides and cholesterol (Kayden & Traber 1993). Bile, produced by the liver, emulsifies the tocopherols incorporating them into micelles along with other fat-soluble compounds, thereby facilitating absorption. Esters of α-tocopherol are hydrolyzed by lipases, and are absorbed as free α-tocopherol. Alfa-tocopherols are absorbed from the small intestine and secreted into lymph in chylomicrons produced in the intestinal wall. Lipoprotein lipases catabolize chylomicrons rapidly and a small amount of tocopherol may be transferred from chylomicrons remnants to other lipoproteins or tissues. During this process, apolipoprotein E binds to chylomicron remnants. Because the liver has specific apoliprotein E receptors, it retains and clears the majority of the chylomicron remnants. Tocopherol in the remnants are bound by VLDL and circulated through the plasma. VLDL is hydrolyzed by lipoprotein lipase to LDL (Papas, 1999) at the mammary epitheilium. α-tocopherol in the milk is mostly associated with the fat-globule membranes (Patton, et al. 1980, Jensen, et al. 1996).

2.3   *Antioxidant Mechanisms of actions*

Antioxidants are increasingly important additives in food processing. Their traditional role is, as their name suggests, in inhibiting the

development of oxidative rancidity in fat-based foods, particularly meat and dairy products and fried foods.

They are called antioxidants because they stop the chemical processes of oxidation of organic molecules. Oxidation reactions can irreversibly change or damage organic materials such as DNA. Free radicals are highly reactive compounds that are created in the body during normal metabolic functions or introduced from the environment. Free radical are inherently unstable, since they contain "extra" energy.

To reduce their energy free radicals react with certain cells in the body, interfering with the cell's ability to function normally. In fact free radicals are believed to play a role in more than sixty different health conditions, including the aging process, cancer, and atherosclerosis. Reducing exposure to free radicals may improve health.

Antioxidants work in several ways: they may reduce the energy of the free radical, stop the free radical from forming in the first place, or interrupt an oxidizing chain reaction to minimize the damage to organic molecules in the body by free radicals.

The body produces several enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase, that neutralize many types of free radicals. Many vitamins and minerals act as antioxidants, such as: vitamin C, vitamin A, vitamin E, carotenoids. Polyphenols, phenols, flavonoids, and other compounds commonly found in the diet have antioxidant properties.

Antioxidants can function by a number of mechanisms. Different enzymes can prevent the formation of radicals or scavenge radicals or hydrogen peroxide and other per-oxides. Other enzymes catalyse the synthesis or regeneration of non-enzymatic antioxidants. Among antioxidant enzymes, superoxide dismutase and catalase have been demonstrated in milk.

Non-enzymatic antioxidants can be formed in the animal body or need to be supplied in the feed as essential nutrients. The iron-binding protein lactoferrin can act as an antioxidant, and vitamin C (ascorbic acid) and vitamin E (tocopherols and tocotrienols) are antioxidant vitamins. Some

carotenoids have provitamin A action but they also have antioxidant functions.

Several non-enzymatic anti-oxidants act as radical scavengers in the lipid phase, such as vitamin E, carotenoids and ubiquinol whereas vitamin C acts in the water phase.

Carotenoids interact with singlet oxygen either via physical quenching mechanisms, in which the excited energy from singlet oxygen is transferred to the carotenoid and then dissipated to the surroundings as heat, or chemical quenching, in which the carotenoid is destroyed in the process by addition of oxygen to its double bond system (Liebler, 1993).

However carotenoids can be degraded and broken into lower molecular weight compounds when they quench singlet oxygen (non enzymatic).

The products of singlet oxygen induced degradation of beta carotene were described by Martin, et al. 1999.

Carotenoids can also be degraded enzymatically by the action of lipoxygenase in plants. This is a very common enzyme and it catalyzes the oxidation of many compounds. There also may be carotenases.

In milk, there is an oxidase enzyme called xanthine oxidase. It is present in high quantity in the milk fat globule membrane. It has been demonstrated that xanthine oxidase can catalyze the degradation of carotenoids (Wache et al. 2002).

The degradation products of carotenoids could produce aromas and flavors in milk. The physical properties (e.g., fat solubility and crystallization) of cis versus trans isomers of β-carotene are different and this may influence their antioxidant activity with cis isomers having higher antioxidant activity (Ben-Amotz and Levy, 1996). Carotenoids are about 3 orders of magnitude stronger in their antioxidant power than flavonoids (Beutner et al., 2001).

Burton and Ingold (1984) found that β-carotene had good free radical trapping capacity to stop the free radical chain reaction mechanism in lipid oxidation at low concentrations of oxygen that are typically found in tissues.

Vitamin E as an antioxidant can be oxidized and then recycled back to its not oxidized form by other antioxidants (Nicholson, & St-Laurent 1991)

while some other antioxidants (e.g., carotenoids) are irreversibly degraded as part of the oxidation process (Hencken, 1992), (Jensen et al.1999).

Tocopherols exhibit antioxidant properties through the phenolic hydroxyl group. This may form a comparatively stable radical upon donating hydrogen to another radical, thereby terminating a free radical chain reaction. This is the basis for the biological activity of the vitamin and is the mechanism responsible for losses of the vitamin during food processing (Oste, et al. 1997).

Definitely Vitamin E and Carotene act by two different mechanism of antioxidant action: Vitamin E is a potent peroxyl radical scavenger (Burton et al. 1989) and can protect polyunsaturated fatty acids (PUFA) within phospholipids of biological membranes (Burton &. Ingold 1983b) and in plasma lipoproteins (Jialal 1995). When vitamin E reacts with a peroxyl radical, it forms α- tocopherol (Serbinova et al. 1991).

2.3   Vitamin E

Vitamin E consists of eight vitamins and α-tocopherol is the major one in bovine milk. Alfa-tocopherol is an important lipid-soluble antioxidant and acts as a radical scavenger. The tocopheryloxy radical formed is relatively stable and can be reconverted into tocopherol by reduction with ascorbic acid.

Different authors have reported concentrations of α-tocopherol between 0´2 and 0´7 mg/l in bovine milk (Jensen, 1995). Gamma-Tocopherol has also been demonstrated and trace amounts of some other vitamins.

Barrefors et al. (1995) reported α-tocopherol levels of 7´4±10´0 mg/g lipid for different herds and also demonstrated low levels of a-tocotrienol.

Lindmark-Ma Ênsson (unpublished results) found 1´0 mg/kg of a-tocopherol in Swedish bulk milk with a mean fat content of 4´3 %.

Colostrum was shown to contain 1´9 mg/l of a-tocopherol decreasing in approximately four days to the level in fresh milk, 0´3 mg/l (Hidiroglou, 1989).

Several reports on the effect of α-tocopherol supplementation on milk oxidative stability have emerged.

Supplementation of cows after one month of lactation with 5 g DL- α-tocopherol intraperitoneally was shown to increase milk α-tocopherol five times and the values remained higher than the original ones for six days (Hidiroglou, 1989). Also intravenously administrated DL- α-tocopherol was found to increase both milk and plasma α-tocopherol levels.

St-Laurent et al. (1990) supplemented Holstein cows for five weeks with 0, 700 or 3000 IU per day of α-tocopherol given in a grain mix. In the group given 3000 IU/day milk α-tocopherol increased from 0´55 to 0´8 mg/l but the level declined to pre-supplementation levels by two weeks after treatment.

In that study, the effects on milk flavour of α-tocopherol supplementation to a feed consisting of grain mix, hay and pasture were also investigated in herds with a chronic spontaneous oxidised flavour milk problem. Alfa-tocopherol supplementation improved milk flavour but there was no relationship between milk α-tocopherol levels and degree of flavour improvement. After the supplementation period all cows got access to spring pasture and then the flavour problem decreased markedly.

The same research group also made a study on supplementation with α-tocopherol together with inorganic selenium mixed in the concentrate ration or alfalfa enriched by spraying with selenium before ensiling (Nicholson et al. 1991). There was no significant effect of α-tocopherol or selenium supplementation on spontaneous oxidation flavour but α-tocopherol inhibited the generation of copper-catalysed oxidised flavour.

The data also suggested that selenium supplementation improved the transfer of dietary α-tocopherol to milk.

Use of higher levels of α-tocopherol supplementation or parenteral injection modes gave more clear improvement of milk oxidative stability (Charmley & Nicholson, 1993; Charm-ley et al. 1993).

Nicholson & St-Laurent (1991) also found that supplementation with α-tocopherol to a corn silage feed was more effective in improving milk oxidative stability than the supplementation to an alfalfa silage feed. The demands on milk oxidative stability and vitamin E levels increase when cows are fed unsaturated fats to modify milk fatty acid composition.

Feeding of rations containing rapeseed were found to increase the proportion of monoenoic fatty acids in milk fat and also its vitamin E content which could explain the prolonged induction time for milk fat oxidation (Flachowsky et al. 1997).

Another group (Focant et al. 1998) added extruded rapeseed and linseed to cow feed, which increased the content of unsaturated fatty acids and vitamin E in milk but decreased its resistance to oxidation.

Supplementation with approximately 10 000 IU of α-tocopherol per day further increased the milk α-tocopherol levels and also the resistance to milk oxidation. An interesting explanation for the difficulties in finding simple relationships between milk α-tocopherol concentration and its susceptibility to oxidation was advanced by Jensen & Nielsen (1996).

At normal rations the content of α-tocopherol per g fatty acid is higher in the milk fat globule membrane than in cream, and the membrane fraction also contains a higher proportion of unsaturated fatty acids. On the other hand γ-tocopherol and b-carotene are only found in cream.

At low concentrations of α-tocopherol in milk fat, however, the decrease in α- tocopherol content is more rapid in the milk fat globule membrane than in cream making the former fraction more susceptible to oxidation.

With respect to storage Vidal-Valverde et al. (1993) found that a-tocopherol in UHT milk stored at 308C decreased by 3±14 % at one month and by 9±30 % at two months.

The losses were marginally higher after storage at 40°C or 50°C.

After storage of UHT milk at -20°C α- tocopherol levels were stable for two months but decreased by 10±21 % after four to eight months.

2.4   Carotenoids

Like tocopherols, carotenoids are fat-soluble compounds and their concentration is influenced by the total fat concentration in dairy products. They function as singlet oxygen scavengers and may also react with other reactive oxygen species.

Herbivorous and omnivorous mammals obtain carotenoids from the diet in grass, leaves, fruits and sometimes flowers For large farm animals, green,

grass, immature and early bloom legumes, and grasses cut and quickly dried into hay provide the major sources of provitamin A.

The most common carotene in all green leaves is β-carotene. Yellow-corn contain β-carotene, cryptoxanthin, some α-carotenoids, γ-carotene, and carotene isomers. Since the dairy cow has a variable carotene intake over the year, as influenced by alternate feeding of fresh pasture and dry roughage and feed, β-carotene and vitamin A content of the milk fluctuates accordingly (Bauernfeind, 1972).

The reality is that degradation products of carotenoids may play an important role in the flavor characteristics of cheeses from all of these species. It just happens that intact β-carotene may have an influence on color in bovine milk and not the milks of sheep and goats.

Cows transfer intact carotenoids to milk and sheep and goat appear not to do this.

The sheep and goats may degrade the carotenoids to other lower molecular weight compounds during the carotenoid absorption process. There is clear evidence that this happens because the Vitamin A content of sheep and goat fat tend to equal to or higher than cow's fat (Yang et al. 1992).

Carotenoid content in rumen is almost the same for all 3 species. Retinol (Vitamin A) content of fat was about same for all 3 species, but β-carotene content is very different.

In vitro trails were conducted with ovine ruminal fluid and it was observed that β-carotene was not destroyed. It is likely that a large quantity of β-carotene degradation product could be found in the sheep and goat fat, but this was not measured in this research (Mora, et al. 1999).

Goat milk contains higher Vitamin A content than cow milk. β-carotene was detected in the colostrum of goat milk but decreased to levels below detection limit in several days. The uptake of β-carotene by the goat is very high, but the degradation of β-carotene at the intestinal level is very efficient to form Vitamin A and other degradation products of β-carotene (Chanda, et al. 1953). It is possible that goats (and sheep) degrade and uptake metabolites of carotenoids that may make important contributions to antioxidant capacity and flavor/aroma in cheese. In the milk from cows and

other species, it is common to find evidence of degradation products of β-carotene.

Lindmark-Ma Ênsson (unpublished results) has found 0´20 mg/kg of β-carotene in bulk milk with a mean fat content of 4´3 %. Ollilainen et al. (1989) found 0´17 mg/kg of β-carotene in whole milk (3´9 % fat) and 0´10 mg/kg in milk with 1´9 % fat. Only traces of lutein and other carotenoids were demonstrated. Khackik et al. (1997) in a detailed study identified thirty-four carotenoids in human milk.

Barrefors et al. (1995) reported β-carotene levels of 3´5± 4´9 mg/g fat in milk with and without off-flavour in different herds.

For some herds β-carotene and/or a-tocopherol levels were lower in milks with off-flavour but this finding was not consistent.

In a supplementation study (Schweigert & Eisele, 1990) a single intravenous or intramuscular injection of 100 or 500 mg b-carotene to cows was shown to increase milk b-carotene from 0´02±0´05 mg/l to 0´13±0´16 mg/l and the maximum was attained approximately one week after injection.

With respect to carotenoid stability during storage Ruas-Madiedo et al. (1998) found that addition of carbon dioxide to milk followed by storage at 48°C for one week after pasteurisation had little influence on the concentrations of β-carotene and α-tocopherol.

Chapter 3

Increasing pasture intakes enhances polyunsaturated fatty acids and lipophilic antioxidants in plasma and milk of dairy cows fed total mix ration

Abstract

PUFAs and liposoluble vitamins in the milk are neutraceutical compouds for their beneficial effects on the human health. The aim of the present study was to evaluate the changes in fatty acid composition and fat-soluble antioxidant content in plasma and milk from cows fed with different percentages of pasture. Cows, from a farm in the Hyblean region sited on mountain level, were randomly divided into three groups (12 animals per group): CTRL fed only a total mix ration (TMR); 30P fed a TMR supplemented with 30% dry matter (DM) from pasture and 70P fed a TMR supplemented with 70% DM of pasture. Blood and milk samples were collected, stored and analysed for content of fatty acids and fat-soluble antioxidants. Fatty acid profile was deeply modified by different diets. CLA, vaccenic acid (VA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) significantly ($P<0.05$) increase in plasma as a function of the proportion of pasture. In agreement with these data, a progressively significant ($P<0.05$) increase in concentrations of VA, CLA and EPA was observed in milk. Such changes in fatty acid composition were accompanied by a concomitant increase in the concentrations of α-tocopherol and β-carotene in both plasma and milk. The increase in EPA, DHA and CLA, β-carotene and α-tocopherol in plasma may have a beneficial impact not only for milk and meat quality, but also for possible beneficial effects on animal health. In particular, an increase of CLA and n-3 PUFA in tissues may result in an increased protection against inflammatory events.

Keywords**:** pasture, plasma, milk, PUFA, fat-soluble vitamins

3.1.   Introduction

Milk and dairy products have always played an important role in human nutrition and, more recently, have also been described as an important source of a variety of relevant biologically-active molecules (Pestana, et al. 2009).

Recently, the potential human health benefits of specific Fatty Acid (FA), including benefits for conjugated linoleic acid (CLA) (Nicholson, et al. 1991) docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) have been identified. CLA comprises a group of unsaturated fatty acid isomers with a variety of healthy biological effects, primarily associated to cis-9, trans-11 CLA and trans-10, cis-12 CLA, (Pestana, et al. 2009).

DHA and EPA are essential for normal growth, brain development, vision and immunity, and also play a vital role in the prevention and treatment of human diseases (Prates & Mateus 2009)

The lipid fraction of dairy products has been often treated as a health concern because of the relatively high content of saturated and trans fatty acids, which adversely influence plasma cholesterol. However, studies have shown that whole milk was more effective in protecting against cardiovascular disease than skimmed milk (Steinmetz et al. 1994).

The reported polyunsaturated fatty acids CLA, EPA and DHA, and fat-soluble-antioxidants α-tocopherol, β-carotene, and retinol, could be envisaged as main players. Changes in plasma and milk content of these compounds are influenced by species, the breed, the parity, the physiological stage, type of diet, the animal production level and sanitary state. However, several reports suggest that the nature of the diet strongly influences the content and the composition of milk fatty acids (Chilliard et al. 2001), and the fat-soluble micronutrient fraction of dairy products, in particular ß-carotene, α-tocopherol and retinol (Martin et al 2004; Kelly et al. 1998; Kelly et al. 1998; Dhiman et al. 1999a).

Supplementing diets with oils, crushed seeds or rumen-protected lipids is the a common nutritional means for manipulating milk fatty acid composition (Chilliard et al. 2002) despite forages often being the major source of fatty acids in the diet (Harfoot & Hazelwood 1998).

On the other hand, ruminant ingestion of plant oils rich in linoleic acid, including soybean oil (Lynch et al. 2005) and sunflower oil (Jones et al. 2005) has increased milk fat CLA concentrations effectively.

Studies have also confirmed that pasture feeding significantly increases milk fat CLA, EPA, and fat-soluble constituents, such as α-tocopherol and β-carotene. Dhiman et al have shown that grazing cows had a 5.7 times higher concentration of CLA in milk than cows fed diets containing preserved forage and grain at 50:50 (Dhiman et al. 2005).

Grass-based diets, especially pasture, also lead to higher milk β-carotene concentrations than diets rich in concentrates or corn silage. The α-tocopherol concentration in fresh pasture is 4-5 times higher than that found in a typical Total Mix Ration (TMR) based on National Research Council (NRC, 2001) (Kristensen et al. 2004) values. However, pasture is unique in terms of increase of PUFAs and fat-soluble antioxidants.

The aim of the present study was to evaluate the changes in fatty acid composition and fat-soluble antioxidant content in plasma and milk from cows fed with different percentages of pasture.

These findings could have an important outcome in terms of maximising the levels of bioactive compounds in milk, keeping the animal management as natural as possible.

3.2. *Materials and Methods*

3.2.1 *Cows and Design*

The experiment was conducted in one farm of the Hyblean region when native pasture was available. This farm had all the typical characteristics of a farmstead cheese producer: native pasture, total mix ration (TMR) facility, and a sufficient number of cows to select a similar stage lactation group for each feeding treatments.

The experimental time was from March to April 2004, with sampling collections every two weeks. Animals started grazing on native pasture about two weeks prior to the first sampling date, in order to allow adaptation of selective behaviour to the nutritional situation.

Daily grazing time was approximately 6 h/d and 16 h/d respectively for each experimental animal group, between the 2 milking times.

Hyblean pasture availability is optimum between February and April determined by the sub-humid Mediterranean climate with mild wet winter and hot dry summers. The most common plants in the Hyblean pasture in terms of occurrence were 39.7% of Calendula (various species), 23.4 % of Geraniaceae (various species), 14.2 % of Graminaceae (various species), unspecified short Asteraceae, and Fabaceae (various species) and other minor families (Carpino, 2003).

In the selected farm 36 Friesian cows were chosen, and randomly divided into three groups. Cows were in late lactation, with a mean Body Weight (BW) of 660 kg, Body Condition Score (BCS) 3.25 and producing an average on 23.93 kg of milk per day with a fat content of about 3.7 % and CP content 3.14%.

Of the three experimental groups one of 12 cows was fed only a TMR (CTRL); another group of 12 cows received some TMR after the evening milking, plus grazing on native pasture approximately for 6 h (30P) starting after the morning milking, and the other group of 12 cows received some TMR after the evening milking, plus grazing on native pasture approximately for 16 h/d (70P) starting after the morning milking.

The dry matter intake of the control group (CTRL) fed only TMR was 21.8 kg/d. The second group of cows (30P) consumed 13.8 kg of DM of TMR and 6 kg of pasture per day. The third group of cows (70P) consumed 5.7 kg of DM of TMR and 12.2 kg of pasture per day.

Total Mix Ration intake was estimated on each sampling day by calculating the differences between TMR offered minus TMR remaining in the morning before milking time; pasture intakes were calculated using Cornell Net Carbohydrate Protein System model (CNCPS) (Fox et al. 1992) elaborating the data for TMR intake as well as chemical analysis parameters for TMR, pasture samples, milk and milk yield for each group. The CNCPS model is in fact based on the energy and protein requirements of the dairy cows correlated to daily milk production and quality (Carpino et al. 2000).

Feed composition and nutritive values of CTRL, 30P, 70P, groups are given in Table 3.1 Effective (NDF, NEL, CP, PS, RUP, NFC) nutrients content of the three diets were also calculated.

Table 3.1 Avarage daily feed consumption data for the cows fed TMR and TMR plus pasture diet.

	TMR (CTRL)	TMR+pasture (30P)	TMR+pasture (70P)
Diet (TMR+pasture) in kg			
Triticale silage	15.2	9.6	4.0
Corn silage	9.1	5.8	2.4
Citrus pulp	9.1	5.8	2.4
Hay graminacee	4.6	2.9	1.2
Soybean F.E. 44	3	1.9	0.9
Soybean roast	0.5	0.3	0.1
Barley	1	0.6	0.3
Corn meal	5.0	3.1	1.3
Megalac	0.2	0.2	0.1
Concentrate supplement	1.5	1	0.4
Intake TMR (DM)	21.8	13.8	5.7
Intake pasture (DM)	--	6	12.2
Diet composition from CNCPS[1]			
NDF[2] (%DM)	39.7	39.34	43
NE_L[3] (Mcal/kg DM)	1.6	1.54	1.3
CP[4] (%DM)	16.1	17.31	16.4
PS[5] (% CP)	27.2	30.1	32.9
RUP[6] (% CP)	37.4	33.82	31.8
NFC[7] (%DM)	34.7	33.17	28.7

CNCPS[1]= Cornell Net Carbohydrate Protein System NDF[2]= Neutral Fiber detergent; NE_L[3]= net energy for
milk production; CP[4]= Crude protein PS[5]= Soluble protein; RUP[6]= ruminal degradable protein;
NFC[7]= non fiber carbohydrate.

3.2.2 Sampling

Cows were milked twice daily. Blood samples from the jugular vein were collected from each cow before milking time in the evening, every two weeks for two months. Samples were collected in evacuated foiled containers containing heparin as anticoagulant, placed on ice, and then promptly transported to the laboratory of CoRFiLaC. They were centrifuged at 2000 rpm 4° C x 10 min in the dark.

Plasma samples were frozen and stored at -80°C to be analysed for fatty acids, fat-soluble antioxidants.

Bulk milk samples from each (cow) group were collected every two weeks for two months and were protected from light, refrigerated immediately and then brought directly to the laboratory of CoRFiLaC where they were split in two portions: the first one was analyzed with milkoscan (MilkoScan Minor, Foss Systems, Hillerød, Denmark) to determine milk fat and protein contents and the second one was stored at -80°C to determine fatty acid profiles, fat-soluble antioxidant content. All the analyses were performed in ice and in the dark.

3.2.3 Chemical analysis: CLA unsaturated fatty acids, retinol, total lipids

Conjugated Linoleic Acid, unsaturated fatty acids, and retinol Total lipids were extracted from plasma and milk by the method of Folch et al (1957).

Aliquots were saponified as described by Banni et al. (2000), in order to obtain free fatty acids (FFA) for HPLC analysis. Particular attention was paid at each stage of analysis to avoid heating or excessive exposure to air and light in order to prevent oxidation of the samples.

Separation of PUFAs was carried out with a Hewlett-Packard 1100 HPLC system (Hewlett-Packard, Palo Alto, CA) equipped with a diode array detector. A C-18 Inertsil 5 ODS-2 Chrompack column, (Chrompack International BV, Middleburg, The Netherlands), 5μm particle size, 150 x 4.6 mm, was used with a mobile phase of CH3CN/H2O/CH3COOH (70/30/0.12, v/v/v) at a flow rate of 1.5 ml/min. Total CLA was detected at 234 nm and at 200 nm the following unsaturated fatty acids: oleic acid (OA),

VA, linoleic acid (LA), total CLA, alpha linolenic acid (ALA), 18:3n6, arachidonic acid (AA), EPA, DHA.

Separation of CLA isomers as free fatty acids was carried out with the same HPLC system using two silver-ion in series ChromSpher 5 lipid Chrompack columns (Chrompack International BV, Middelburg, the Netherlands), 5-mm particle size, 250x34.6 mm; the mobile phase was n-hexane with 0.5% ether and 0.1% CH3CN at a flow rate of 1 ml/min. Plasma and milk retinol were measured as previously described (Banni, et al. 1999). Retinol was determined simultaneously from aliquots of total lipid extract, using a Hewlett-Packard 1100 liquid chromatograph equipped with a diode array detector. An Inertsil ODS-3 Chrompack\column (Chrompack International BV), 5 mm particle size, 150x3 mm was used with 100% methanol as a mobile phase, at a flow rate of 0.7 ml/min. Retinol was detected at 324 nm. UV spectra of the eluate, generated by the Phoenix 3D HP Chemstation software, was obtained at every 1.28 s and electronically stored. The spectra were taken to confirm the identification of the peaks.

3.2.4 β-Carotene

The extraction of β-carotene from plasma and milk was performed as indicated by Palozza et al (1992a). Sample was dissolved in methanol, and 20-μl aliquot was analysed by reverse phase HPLC with spectrophotometric detection on a Perkin-Elmer LC- 295 detector at 450 nm (β-carotene content) and at 350 nm (β-carotene 5,6-epoxide).

The column was packed with Alltech C18 Adsorbosphere HS material, 3-μm particle size, in a 15 x 0.46-cm cartridge format (Alltech Associates, Deerfield, IL). A 1-cm cartridge precolumn, containing 5-μm C18 Adsorbosphere packing was used. Analyses were done by gradient elution, the initial mobile phase was 85% acetonitrile/15% methanol, with the addition at 8 min of 30% 2-propanol. Ammonium acetate, HPLC grade, 0.01%, was added to the initial mobile phase.

3.2.5 α-Tocopherol

Plasma tocopherol was extracted according to Palozza et al. (1998) where 500 μL of plasma were mixed with 500 μL of distilled water and extracted with ethanol 1 ml and 3 ml of hexane.

Both α-tocopherol and the internal standard (tocopherol acetate; 150 μL of a 60 μg/ml ethanol solution) contained in the hexane phase was extracted by centrifugation (10 min at 710 x g). A second extraction with 3 ml of hexane was subsequently performed. Concentration of α-tocopherol in milk was determinate according to Noziere et al (2006a). Two ml of milk were combined in a test tube with BHT, 14.6 ml of saponification solution 11% KOH (wt/v), 55% ethanol (v/v) and 45% deionised water (v/v) plus 0.4 ml of internal standard: 2.25% α-tocotrienol (wt/v); 97.75% ethanol (v/v). The tubes were then placed in a shaking water bath for 20 min at 80°C.

After cooling in an ice water bath for 10 min, α-tocopherol was extracted using a hexane and water mixture (2/1, v/v). The hexane phase, of both plasma and milk samples, was evaporated under a stream of N2 and redissolved in 60 μL methanol and a 20 μL aliquot was analysed by reverse phase HPLC with fluorescence detection on a Perkin Elmer 650-LC fluorescence detector with excitation at 295 nm and emission at 340 nm. α-tocopherol was eluted with 100% methanol on an Alltech C18 3 μm column (Alltech Associates, Deerfield, IL).

3.3 Statistical Analysis

Statistical analysis was carried out using the SAS-software GLM procedure (SAS, 1999). Data for PUFA's and fat-antioxidants in plasma and milk sample were analysed using ANOVA model with factorial term for diet composition. Means were tested for differences between diets using t-test (LDS, $P < 0.05$).

3.4. Results

The results clearly show a correlation between amount of native pasture in the diet and level of fatty acids and fat-soluble antioxidants in plasma and milk produced from grazing animals. Table 3.2 shows the results

of fatty acids contents in plasma in the three groups. In particular, total CLA increased almost two- and three-fold in 30P and 70P, respectively, compared to CTRL as a function of the proportion of pasture intake. In parallel, same pattern was found for ALA in plasma samples.

In addition, VA increased slightly in 30P (17%) and much more in 70P (80%) compared to CTRL. Linoleic acid showed a slight increase (16%) only at higher pasture intake compared to CTRL. A progressive significant increase was observed for EPA and DHA in 30P and 70P. In contrast no significant differences in AA concentrations were found among three groups.

Table 3.3 shows the results of fatty acids contents in milk in the three groups. A progressive significant increase of two- and three-fold was only observed for ALA, in 30P and 70P, respectively, compared to CTRL. Oleic acid, VA, LA and total CLA increased on average 20% and 55% in 30P and 70P, respectively, compared to CTRL.

Arachidonic acid significantly increased at both pasture intake versus CTRL, whereas EPA significantly increased at higher pasture intake versus CTRL.

Moreover, the ratio between total CLA and VA calculated for the three groups of samples was much lower in plasma than in milk.

Table 3.2 and 3.3 show the content of fat-soluble antioxidants in plasma and in milk. A progressive increase of β-carotene and α-tocopherol was found in cow plasma by enhancing the pasture, whereas no variation for retinol (Table 3.2). In contrast, in milk samples β-carotene significantly increased at lower pasture intake compared to CTRL without significative differences between 30P to 70P, whereas retinol and α-tocopherol significantly increased at higher pasture intake versus CTRL (Table 3.3).

Table 3.2 Fatty acid and fat-soluble antioxidant profile in plasma.

	Fatty acid groups (mg·g⁻¹)					
	CTRL	SEM	30P	SEM	70P	SEM
OA[1]	36.87[c]	1	43.37[b]	1.69	48.85[a]	2.65
VA[2]	59.0[c]	2.05	73.75[bc]	2.07	106.90[a]	3.03
LA[3]	289.89[b]	48.98	268.07[b]	108.30	311.27[a]	34.01
Tot CLA[4]	0.40[c]	0.21	0.70[b]	0.20	1.04[a]	0.21
ALA[5]	0.24[c]	0.11	0.41[b]	0.12	0.75[a]	0.12
GLA[6]	0.24	0.01	0.27	0.03	0.22	0.01
AA[7]	0.17	0.03	0.15	0.02	0.20	0.02
EPA[8]	2.47[c]	0.80	3.90[b]	0.99	6.10[a]	1.56
DHA[9]	13.36[c]	1.56	19.82[b]	0.56	29.83[a]	2.56
	Fat-soluble antioxidants (µg·mL⁻¹)					
β-carotene	1.74[c]	0.35	2.89·[b]	0.38	4.39[a]	0.45
Retinol	0.41	0.10	0.53	0.08	0.46	0.22
α-tocopherol	3.32[c]	0.37	4.55[b]	0.2	5.60[a]	0.5

[a c] Means not sharing the same superscript are different ($P \leq 0.05$).
NS= not significant;
[1]OA= oleic acid
[2]VA= vaccenic acid.
[3]LA= linoleic acid
[4]Tot CLA= conjugated linoleic acid.
[5]ALA= alpha-linolenic acid
[6]GLA= gamma-linolenic acid
[7]AA= arachidonic acid
[8]EPA= eicosapentaenoic acid.
[7]DHA= docosahexaenoic acid.

Table 3.3. Fatty acid and fat-soluble antioxidant profile in milk.

	Fatty acid groups (mg·g⁻¹)					
	CTRL	SEM	30P	SEM	70P	SEM
OA[1]	202.00 [b]	16.06	237.94 [b]	16.06	323.26 [a]	14.67
VA[2]	7.32 [b]	0.70	8.96 [ab]	0.70	11.56 [a]	0.65
LA[3]	24.30 [c]	1.0	29.68 [b]	1.18	35.23 [a]	1.08
Tot CLA[4]	3.6 [b]	0.31	4.2 [b]	0.3	5.6 [a]	0.28
ALA[5]	4.1 [c]	0.32	8.6 [b]	0.32	11.98 [a]	0.29
GLA[6]	0.16 [c]	0.06	0.53 [a]	0.06	0.38 [b]	0.05
AA[7]	0.085 [b]	0.03	0.22 [a]	0.03	0.18 [ab]	0.03
EPA[8]	0.41 [b]	0.04	0.49 [b]	0.04	0.81 [a]	0.04
DHA[9]	NS		NS		NS	
	Fat-soluble antioxidants (µg·g⁻¹ of lipids)					
-carotene	3.02 [b]	0.5	15.21 [a]	0.5	16.18 [a]	0.8
etinol	2.50 [b]	0.4	2.71 [b]	0.6	3.50 [a]	0.2
-tocopherol	12.9 [b]	0.2	13.20 [ab]	0.5	16.05 [a]	0.5

[c] Means not sharing the same superscript are different ($P < 0.05$).
NS= not significant;
[1]OA= oleic acid
[2]VA= vaccenic acid.
[3]LA= linoleic acid
[4]Tot CLA= conjugated linoleic acid.
[5]ALA= alpha-linolenic acid
[6]GLA= gamma-linolenic acid
[7]AA= arachidonic acid
[8]EPA= eicosapentaenoic acid.
[7]DHA= docosahexaenoic acid.

3.5.   *Discussion*

The results clearly showed that fatty acid composition and fat-soluble antioxidant content in plasma and milk from cows were deeply influenced by the percentage of pasture in the diet. In particular, a remarkable increase in the content of CLA and VA was observed in plasma and milk following an increased ratio pasture/TMR.

Many authors report that milk fat from cows grazing pasture show a CLA increase compared to milk fat from cows fed by dry forage (Dhiman, et al. 1999a). Dhiman et al (2000) reported that cows grazing pasture had 3 times higher CLA content in milk fat (2.21% of total FA) compared to cows fed a diet containing 50% conserved forage (hay and silages) and 50% grain (0.38% of total FA). It has been also tested that milk fat CLA increased linearly as the proportion of fresh pasture increased in the dairy cow diet (Banni, et al. 1999; Kelly, et al. 1998; White, et al. 2002). Fresh grass contains approximately 1 to 3 % FA on a DM basis, depending on the variety of the pasture, with the highest FA contents usually occurring in the spring and fall seasons (Dhiman, et al. 1999a).

Ruminant feeds are rich in PUFA such as LA and ALA that are rapidly hydrogenated by rumen bacteria to produce more highly saturated end products. However, ALA is the predominant fatty acid in fresh pasture, representing 48 to 56% of total fatty acids, and in the bovine rumen may contribute to VA production. (Kay, et al. 2005). Moreover, changes of VA and CLA content in milk also depend on other factors such as ruminal pH and bacteria population (Jenkins, 1993; Britton, 1995; Kellens, et al. 1986). It is known that cows grazing on pasture have a higher ruminal pH that is favourable for cellulolytic bacteria growth in the rumen, responsible for CLA and VA production (Martin & Jenkins 2002).

In the current study the ratio CLA/VA was much lower in plasma than in milk, suggesting the important contribution of plasma VA to CLA synthesis in the mammary gland by the epithelial tissue Δ9-desaturase (Griinari, et al. 2000).

In fact, it is known by literature that CLA in milk originates as an intermediate product from either ruminal biohydrogenation of ALA and LA

and from the endogenous synthesis in mammary gland that is the major pathway of CLA synthesis from VA in dairy cows (Dhiman, et al. 1999a).

According to this theory of endogenous synthesis of CLA, our results showed a higher content of CLA in milk than in plasma (Griinari and Bauman 1999; Griinari, et al. 2000.

Besides to the increase of total CLA and VA in plasma and milk from cows with higher intake of pasture, our data also show a consistent increase of ALA and EPA. LA (n-6) and ALA (n-3) are the main PUFAs in milk and the precursors respectively of AA and of EPA and DHA that are further converted to eicosanoids. In particular, EPA is an important long n-3 fatty acid because is able to inhibit the conversion of n-6 fatty acids to harmful eicosanoids, thereby protecting against cardiovascular diseases and cancer. In this study the increase of n-3 fatty acids (ALA and EPA) especially in milk derived from group 70P shows the importance of fresh pasture on these molecules that have a beneficial impact on human health.

Thus, our results show how the amount of fresh pasture affects the content of total CLA and the ratio n-6 and n-3 in milk, and also the content of OA that is considered to be favourable for health. According to the above considerations, milk derived from grazing cows may be considered a healthy food better that any other one because is rich in OA, in total CLA and has a low ratio n -6 and n-3.

Studies have shown that enhancing the PUFA content of plasma and milk is associated with increased susceptibility to auto-oxidation and development of milk off-flavours (Kristensen, et al. 2004). PUFAs in plasma and milk are protected from oxidation by natural antioxidants. Among the fat-soluble antioxidants, the most important are α-tocopherol and β-carotene, the precursor of retinol. Alpha-tocopherol is a potent peroxyl radical scavenger and it can protect polyunsaturated fatty acids within phospholipids of biological membranes and in plasma lipoproteins (Burton, 1989).

The plasma levels of α-tocopherol and β-carotene are extremely dependent on the diet. In the present study, the plasma concentrations of the fat-soluble α-tocopherol and β-carotene differed between pasture and TMR-fed cows.

Plasma concentrations of α-tocopherol and β-carotene were significantly increased by enhancing dietary pasture/TMR ratio (30P vs 70P), suggesting that pasture naturally supplies high levels of both antioxidants in cows. It has been reported that α-tocopherol concentration in fresh pasture (approximately 106 i.u./kg DM) is 4-5 times greater than that found in a typical TMR based on NRC (2001) values (15 i.u./kg) (NRC, 2001). Similarly, native pasture is very rich in plants that contain β-carotene and especially Calendula (Carpino, 2003) that is very represented in our native pastures.

The progressive increase of the two fat-soluble antioxidants in plasma observed by increasing dietary pasture/TMR ratio is particularly interesting in view of the possible metabolic interactions occurring among the fat-soluble antioxidants. It has been shown that plasma α-tocopherol concentrations were depressed in animals supplemented with β-carotene (Palozza & Krinsky, 1992a) or vitamin A (Nonnecke, et al. 1999) by mechanisms involving competition in intestinal absorption (Frigg & Broz, 1984) or oxidative interactions (Palozza & Krinsky, 1992a). Our results indicate that pasture obviously provides high amounts of both α-tocopherol and β-carotene in plasma for milk synthesis.

Although plasma β-carotene concentration was remarkably higher in pasture-fed cows, plasma retinol concentrations did not statistically exceed those in TMR-fed cows. Our results and those from studies in humans (Mayne, et al. 1998) in which β-carotene was given as a supplement, indicate that an increase in plasma of β-carotene concentrations has minimal impact on plasma retinol concentrations.

Chew et al (1993) also reported that oral administration of β-carotene to calves fed a diet containing normal concentrations of vitamin A does not affect plasma retinol concentration.

Even though transport of α-tocopherol and β-carotene from plasma lipoproteins into mammary gland conforms to Michaelis-Menten kinetics, concentrations of α-tocopherol and β-carotene in milk are thought to be a function of the dietary intake (Focant, et al. 1998). In agreement with this, in the present study, the concentrations of α-tocopherol and β-carotene in milk, paralleled those observed in plasma. A clear relationship between plasma

concentrations and secretion into milk of α-tocopherol and β-carotene has been also reported by other authors (Hidiroglou, et al. 1989; Nicholson & St-Laurent 1991).

The concentrations of the two fat-soluble antioxidants in milk derived from pasture supplemented groups were much higher than the minima suggested by Al-Mabruk et al to insure a favourable oxidative stability of milk (Al-Mabruk et al. 2004)

Milk from cows fed a higher percentage of pasture (70P) had a more favourable fatty acid pattern, high total CLA and n-3 PUFA content and lower n-6/n-3 ratio, than milk from cows fed a conventional TMR diet. This may have several implications in the prevention of mastitis and immune depression in cows (Overton & Waldron, 2004). On the other hand, changes in milk fatty acid composition and fat-soluble antioxidant concentration may have several implications for nutrient content, organoleptic attributes and shelf life of milk.

3.6. *Conclusion*

In conclusion, our study shows that native pasture increases CLA, n-3, α-tocopherol and β-carotene levels in both plasma and milk.

The observed variations in milk may have a great potential for human consumption as a source of PUFAs with important beneficial activities. Taking into account that ewe milk is generally used for cheese making and that milk transformation does not influence the CLA content of dairy products (Shantha, et al. 1995) pasture management could become a useful strategy to naturally manipulate dietetic characteristics of milk and dairy products.

Further studies are needed to verify whether changes in fatty acid profile and other lipid soluble compounds in milk could be used as quantitative and qualitative biomarkers of PDO (products with Denominations of Origin).

Chapter 4

Alpha and gamma tocopherol content in milks from different species collected in summer time in South-eastern Sicily

Abstract - Vitamin E is a very important antioxidant for oxidative stability of milk and for human health, minimizing lipid oxidation. Its concentration in milk depends on several factors as species, feed, season, bioavailability of stereoisomers, PUFA content and animal's health status. The purpose of this study was to determine the content of α-tocopherol and γ- tocopherol in milk by High Performance Liquid Chromatography method during summer time. Milk samples from different species (buffalo, cow, goat and sheep) have been collected between June and July from five farmhouses located on hyblean highlands of South-eastern Sicily. In the present investigation the discrepancies found about the levels of α-tocopherol in our milk samples could be explained with interspecies variability and the similarity could be the consequence of a combination of feed characteristics. Although α-tocopherol content was within the range reported in literature, the low concentrations might be explained by poor pasture in α-tocopherol in summer and by vitamin decreases in animal's plasma and in milk in answer to a major heat stress. However, ewes' milk had higher significantly levels and buffalo's milk lower ($p<0.05$) of α-tocopherol than other milk varieties. We cannot compare our results about γ-tocopherol content in milks because lacking data in literature, although its beneficial roles in human health and in protecting foods. In this study differences species-specific have been found with a higher significantly content ($P < 0.05$) of γ isomer in goat and buffalo milk compared to other milk varieties.

Milk/ species/ alpha tocopherol/ gamma tocopherol/ summer

4.1 Introduction

Although cow milk remains widely consumed as drink by humans, in recent decades an increasingly interest is born for the milk from other mammalian species as a valuable alternative source to cow's milk especially in the treatment of various metabolic disorders (Huppertz et al, 2006).

Milk is a very complex food matrix whose lipid fraction is that mostly variable, particularly influenced by genetic factors and environmental conditions including breed, number and stage of lactation, climate, feed (Reynolds et al, 2008; Slots et al, 2006). Differences in fat percentage are found both inter-species with about 4% in cow's milk to more than 8% in buffalo's milk and intra- species (Lucas et al, 2008; Morales et al, 2000).

The milk fat globule membrane (MFGM) is rich in polyunsaturated fatty acids (PUFA) that are particularly sensitive to lipid oxidation, responsible for off-favour development in milk, thus, the balance between pro and anti-oxidative components is important for oxidative stability of milk. If by one side the fresh green pasture enriches the milk in linolenic acid- ω-3 content with consequently lowering of ω-6/ ω-3 ratio, by other side the milk needs enough vitamin E as powerful antioxidant for inhibition of lipid oxidation reactions and in particular for stabilization of ω-3 fatty acids which are more oxidatively vulnerable than ω-6 (Havemose et al, 2006; Liesegang et al, 2008). The vitamin E content in milk depends on species, season, feed, geographical location and lastly no because less important on analytical method used to measure its concentration.

In general, the term vitamin E represents a family of eight natural compounds α, β, γ, δ- tocopherols and tocotrienols that differ in the number and in the position of methyl groups substituted on the chromanol ring. The plants are the only organisms able to biosynthesize the various isomers of vitamin E whose concentration depends on stage of growth but also on genetic factors, season, access to water, days of sunshine, harvesting methods, processing, and storage conditions (Kay et al, 2005; Osuna-Garcia et al, 1998). Hay and silage contain from 20 to 80% less vitamin E than fresh green forages (Kay et al, 2005). However, the concentration of vitamin E in fresh forage decreases rapidly with cutting, silage and exposure to sunlight (Kay et al, 2005).

Humans and animals don't have the ability to synthesize tocopherol isomers that obtain from dietary sources or supplements (Bramley et al, 2000). The α and γ isomers of vitamin E, the most representative molecules, although they do not compete during intestinal absorption, are kept at different concentrations in human and animal plasma and tissues (Wagner et al, 2004). Moreover, it has been observed a different bio-availability for the α-tocopherol stereoisomers when humans' and animals' diet is supplemented with synthetic vitamin E (all-rac-α-tocopherol), depending on species and within species by animal's metabolic status and tissue studied (Slots et al, 2007). In particular, the natural stereoisomer, RRR-α-tocopherol, is that preferentially transferred from feed to plasma, tissues and milk than all others 7 stereoisomers (Meglia et al, 2006). The MFGM fractions seem favour absorption and transportation of vitamin E by chilomicrons (Bezelgues et al, 2009). Thus, Spitsberg considered MFGM as a putative delivery system for microelements including liposoluble vitamins (Spitsberg, 2005).

The most of information we have is limited only to α-tocopherol that has been studied extensively in cow's milk but less in that from other species. In fact, the α- tocopherol has been always considered as the most powerful antioxidant than other isomers in inhibiting free radical-induced lipid autoxidation but, new evidences suggest important roles also for γ-tocopherol including protection against nitrogen free radicals associated to degenerative brain disorders and a more stable and more efficient antioxidant effect in food lipids than α-tocopherol (Wagner et al, 2004). In addition, it has been found that γ- tocopherol is a good antipolymerization agent in thermoxidation conditions and a better stabilizing agent of emulsions (Wagner et al, 2004). The lack of one methyl group in γ-tocopherol allows to be more efficient in different locations than α-tocopherol.

Furthermore, it has been discovered that dietary intake of only α-tocopherol may lower γ– tocopherol concentrations in plasma and tissues, thus a major consideration should be given to supplementation with combined α -and γ– tocopherols to have a wider beneficial synergistic effect (Handelman et al,1985).

In a context where consumers' expectations for a high quality natural food is crucial, it is important to have major information about nutritional components of milk from different species. The aim of this work was to determine the content of vitamin E in milk from different species collected in extreme conditions during summer time in Sicily, season characterized by hot temperature and by drought. In fact, it is known from literature that dairy animals exposed to hot environment temperature have lower levels of vitamin E in plasma and in milk in answer to a major heat stress (Hala et al, 2009; Harmon et al, 1997). Heat stress generally leads to oxidative stress increasing mastitis frequency and somatic cells count (SCC) in milk so it is needful to supplement their diet with antioxidant vitamins such as vitamin E (Megahed et al, 2008). In addition, several authors have reported a correlation between vitamin E concentration and somatic cell count (SSC) in milk, as an indicator of animal health status (Baldi et al, 2000; (Weiss et al, 1996).

Furthermore, in the absence of available data in literature on concentrations of γ tocopherol in milk from different species, the aim of the present study was also an updating of nutritional tables on content of this isomer in milk because its beneficial effects on human health.

4.2. Material and methods

4.2.1. Approach

The experiment was carried out during summer season, between late June and early July, 2008. The mean temperature recorded in that period was 26°C (minimum, 19°C; maximum, 32°C). Milk samples from Buffalo, Friesian cow, Modicana cow, goat, and Comisana sheep were respectively collected from five farmhouses, located on hyblean highlands of South-eastern Sicily.

A standardized questionnaire was submitted to farmers in order to collect information about the stage of lactation of animals, number of daily milking and feeding that was essentially characterized by TMR (total mix ratio) ad libitum and what was available in the field as fresh forage.

Milk samples were collected from the morning milking in bottles of 500 mL, wrapped in aluminium foil to protect from light, and quickly

transferred in refrigerated conditions to CoRFiLaC's laboratories for analysis. Milk collection was repeated twice a week for a total 3 weeks period.

4.2.2 Chemical analysis

Milk samples from different species were analysed for fat, protein, lactose contents by MilkoScan ™ Minor (FOSS, Italy) and total milk Somatic Cell Counts (SCCs) were determined by Fossomatic™ Minor (FOSS Integrator, Italy).

4.2.3 Determination of vitamin E

The extractions of α and γ-tocopherols were performed on fresh milk samples in darkness, to avoid oxidations due to daylight.

Each milk sample (1mL) was mixed with 2 mL of ethanol (99.5% v/v) containing BHT (0.5%w/v) and 1 mL of saponification solution (KOH 10%w/v). The dispersion was incubated in a water bath at 65°C for 45 min after being purged with nitrogen and capped. During the incubation, the tubes were briefly vortex-mixed every 10 min. After cooling of the samples on ice, 4ml hexane was added and the dispersion centrifuged at 1500xg for 10 min at 10°C. The upper organic layer was carefully transferred to a clean Pyrex tube. Another 3 mL of hexane was added and centrifuged again. The hexane solvent isolated combined with the previous one was evaporated under a gentle stream of nitrogen and the residue reconstituted in methanol. The solution was transferred into amber vial and analyzed by HPLC method by using a SB-C18 column (5 µm particle size, 4.6 nm ID x 250nm, Agilent Zorbax) and a mobile phase of methanol (100% v/v). The HPLC system (Waters 2695) was equipped with a multi λ fluorescence detector (Waters 2475) using an excitation of 297 nm and an emission of 340 nm for tocopherol isomers detection.

The concentrations of α and γ-tocopherol in the samples were calculated with external standards through a linear regression from known standards.

4.2.4 Determination of linoleic and linolenic acids

Milk samples were stored at –80°C until analysis. Linoleic (LA) and linolenic acids (LNA) were determined by reversed-phase High Performance Liquid Chromatography (HPLC) method described by Banni et al (1994). A SB-C18 column (5 µm particle size, 4.6 nm ID x 250nm, Agilent Zorbax) was used with a mobile phase of CH3CN/H2O/CH3COOH (70:30:0.12 v/v/v) at a flow rate of 1.5 ml/min. The HPLC system (Waters 2695) was equipped by dual λ adsorbance detector (Waters 2487) and LA and LNA were detected at 200 nm.

4.3 Statistical Analysis

The milk samples were analyzed in duplicate. All data were presented as means ± standard deviations (s.d.). Differences between the groups of animals were analyzed by analysis of Variance using Statistical software program (SigmaStat, version 3.5). The level of significance was taken as $P < 0.05$.

## 4.4 Results and discussion

4.4.1 Tocopherol isomers in milk

The predominant isomer detected was α– tocopherol in all milks analyzed from the different species. The means ± s.d. of α- tocopherol levels in the different types of milk, calculated respectively as µg.mL-1 and as µg.g -1 of total lipids of milk, are reported in figures 4.1A and 4.2 A.
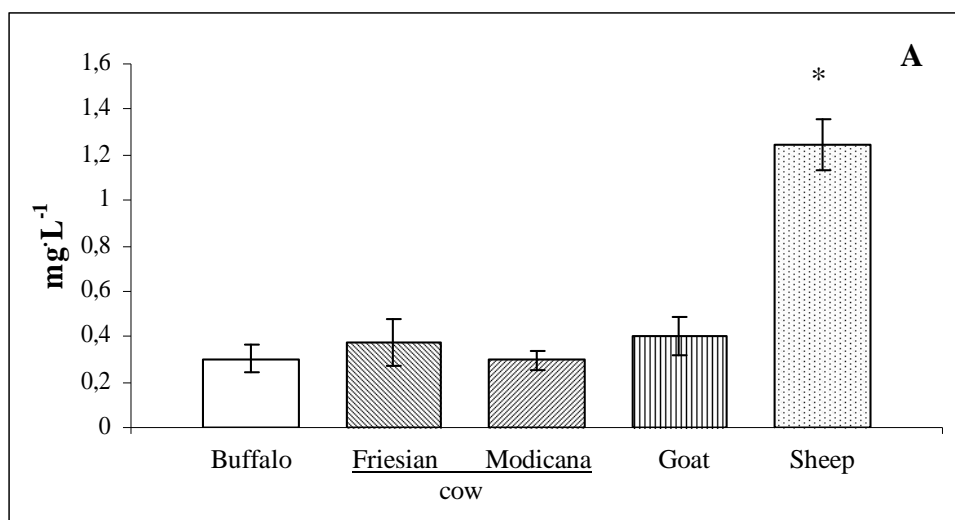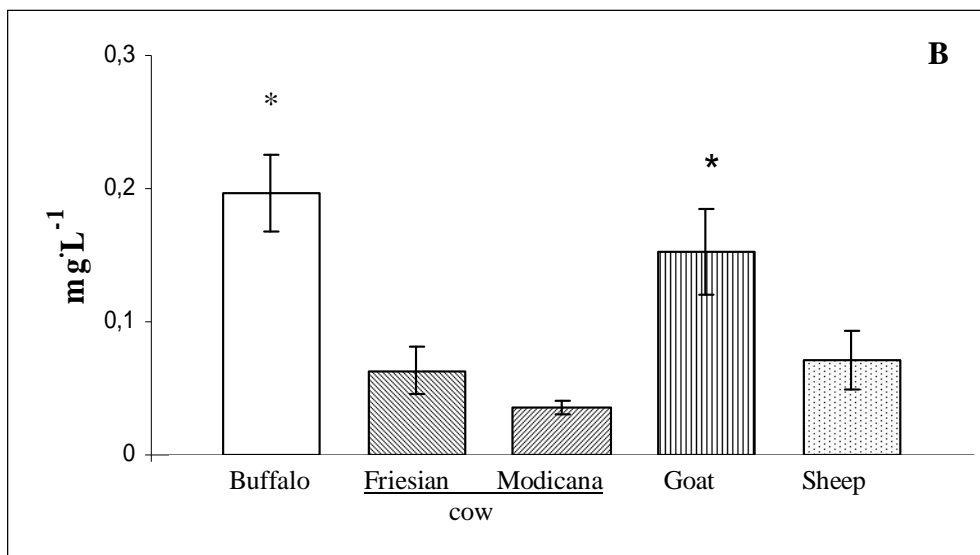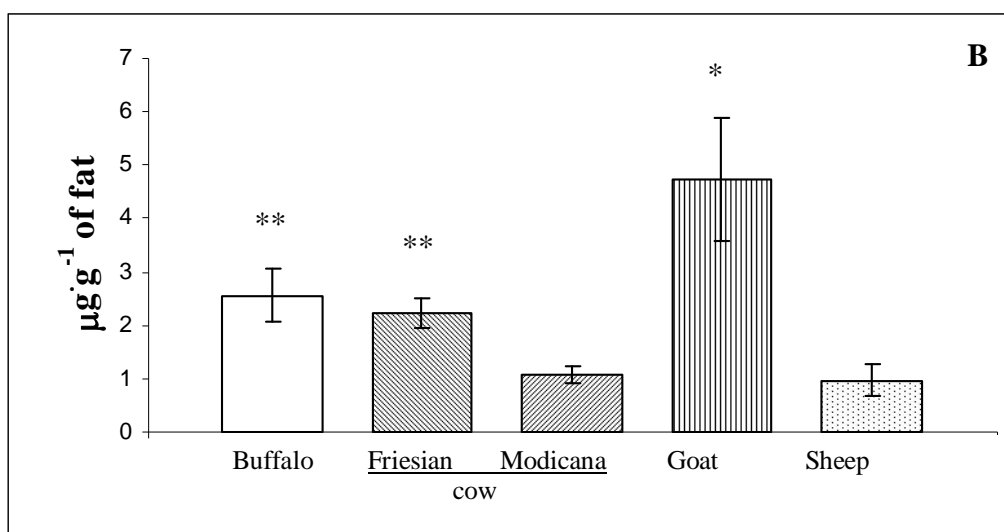
Figures 4.1. Content of α-tocopherol (**A**) and γ-tocopherol (**B**) in milk from different species. Each sample was analyzed in duplicate and each bar represents the mean ± s.d. of six experiments. Tocopherol concentrations are expressed as mg·L^{-1}. Significant differences are shown as * ($P < 0.05$).

The levels of α-tocopherol were significantly higher in ewes' milk sample (P < 0.05) in comparison to other milk varieties when calculated respectively as µg.mL-1 and as µg.g -1 of total lipids of milk. In contrast, buffalos' milk, when α-tocopherol levels were calculated on total lipids, showed a lower significantly content (P <0.05) than other species due to a higher content of fat. A statistically significant difference (P < 0.05) on α-tocopherol content when calculated on total lipids was also found between Modicana and Friesian cows' milk samples (Figure 4.2A).
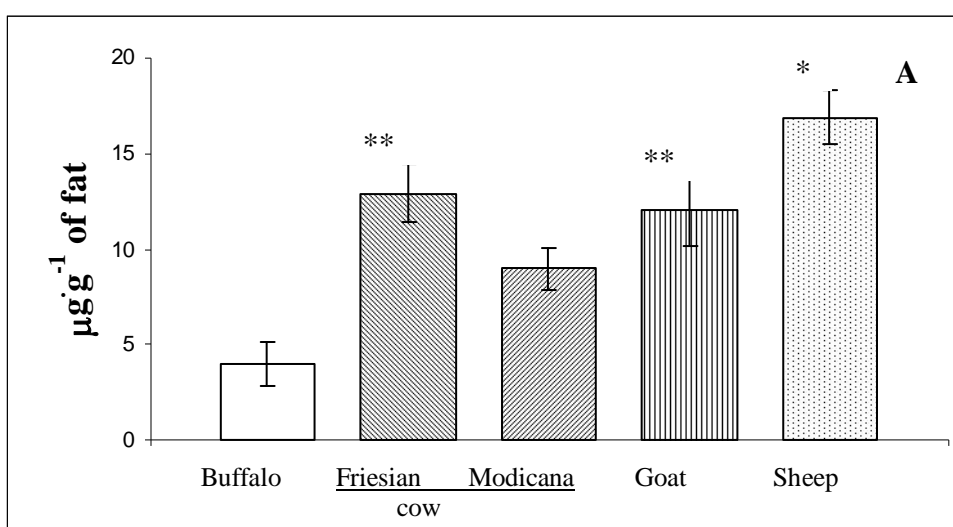
Figures 4.2 A Content of α-tocopherol in milk from different species. Each sample was analyzed in duplicate and each bar represents the mean ± s.d. of six experiments. Tocopherol concentrations are expressed as µg·g^{-1} of fat. Significant differences are shown as * and ** ($P < 0.05$).

A different pattern has been found for γ-tocopherol in milk samples. The means ± s.d. of γ- tocopherol levels in the different types of milk, calculated respectively as μg.mL-1 and as μg.g -1 of total lipids of milk, were reported in figures 1B and 2B. The levels of γ- tocopherol (μg.mL-1) were significantly higher in buffalo's and goat's milk (P < 0.05) compared to other species (Figure 4.1B). In contrast, when the results were reported on total lipids (μg.g -1), γ- tocopherol levels in goat's milk were significantly higher (P < 0.05) compared to other species (Figure 4.2.B).

Figures 4.2.B Content of γ-tocopherol in milk from different species. Each sample was analyzed in duplicate and each bar represents the mean ± s.d. of six experiments. Tocopherol concentrations are expressed as $\mu g \cdot g^{-1}$ of fat. Significant differences are shown as * and ** ($P < 0.05$).

A statistically significant difference (P < 0.05) on γ- tocopherol content was found between Modicana and Friesian cows' milk samples (Figure 4.2.B). The relative levels of α- and γ-tocopherol were also calculated as percentages of the total tocopherol (Figure 4.3). The mean (and range) as percentage values for α-tocopherol was significantly higher (P < 0.05) in ewes' milk with 94.0% (91.7 to 95.0) than other milk samples. The means (and ranges) as percentage values for γ-tocopherol were significantly higher (P < 0.05) in buffalo's milk with 39.5% (34.8 to 43.7) and goat's milk with 27 % (20.8 to 34.6) than other milk samples.
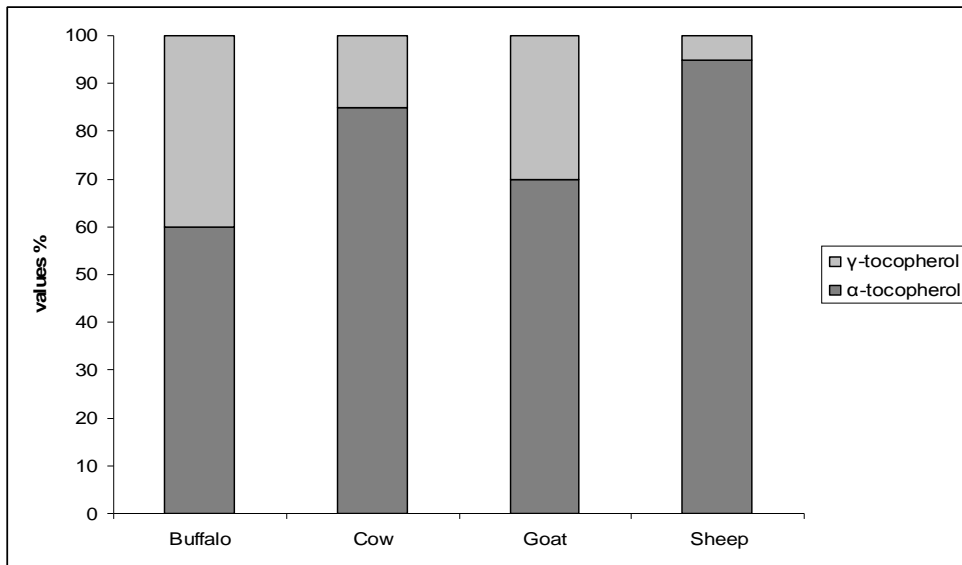
Figure 4.3: Tocopherol isomers average composition (%) in milk from different species.

In general the α- tocopherol concentrations in our samples agreed to those reported in literature (Bergamo et al, 2003; Raynal-Ljutovac et al, 2008; Liesegang et al, 2008) for animals TMR- fed, with the exception of buffalo's milk which had α- tocopherol levels lower than those reported in literature (Balestrieri et al, 2002; Panda and Kaur, 2007). Although buffalo's milk has a content of fat twice as high as in bovine milk and is lacking of β carotene, some authors sustain that buffalo's milk has a major resistance to lipid oxidation than bovine milk (Balestrieri et al, 2002). However, Panda and Kaur concluded that buffalo's milk with α tocopherol levels ranged between 1.38 and 1.61 µg.mL-1 and between 20.55 and 25.56 µg.g -1 of total lipids of milk (about 5 - 6 times higher than our results) showed slight oxidation (Panda and Kaur, 2007).

The levels of α-tocopherol levels in cows' milk were in the range reported by several authors (0.2 and 0.7 mg.mL-1) (Bergamo et al, 2003; Weiss and Wyatt, 2003). In our study the α–tocopherol concentration in Friesian milk was very similar to that reported by Havemose for cows grass clover silage and hay fed (Havemose et al, 2006). However, about the ideal concentration of α-tocopherol to improve the oxidative stability of bovine milk, conflicting results have been showed by several studies with values ranged between 0.6 and 2.7 mg.L-1 (Al-Mabruk et al, 2004; Charmley and Nicholson, 1994; Focant et al, 1998).

Considering α–tocopherol in small ruminants, the concentration in ewes' milk was twice and half as high compared to goats ($P < 0.05$). These values found in the present study were consistent with those determined by Liesegang in milk of sheep and goat fed same diet composition, confirming a different vitamin E- related metabolism between the two species (Liesegang et al, 2008). However, α–tocopherol content in goat's milk was lower than that reported by Kondyli in goat's milk from Greek breed (Kondyli et al, 2007). Studies report that sheep have a great capacity to store vitamin E mainly in the liver during phase of sufficient supply (Fry et al,1996). This might explain why the content of α-tocopherol in our ewes' milk was enough high in summer without extra supplementation. Some authors (Atwal et al, 1990; Havemose et al, 2006) suggested that increasing the proportions of linolenic acid with the feed, increased also the transfer of α-tocopherol to the milk. However, the enrichment of milk lipid fraction of α-tocopherol might be also limited by availability of plasma lipoproteins carrying α- tocopherol to the mammary gland (Wagner et al, 2004).

We cannot compare our results about γ-tocopherol content in milk samples because lacking data in literature, little attention in fact, has been drawn to this isomer compared to α–tocopherol. However, in our study differences species-specific have been found with a higher content of γ isomer in milk from goat and buffalo compared to other varieties.

It is known from literature that α -tocopherol might become pro-oxidative to high concentrations (Havemose et al, 2006), in contrast γ-tocopherol should work better as antioxidant (Wagner et al, 2004). Thus, the combination of both forms might provide superior oxidative protection to food thereby reducing the amount of free radicals introduced by human diet.

It should be noted that in the present study the milk samples have been collected between late June and early July in Sicily, period characterized by poor quality pastures with low levels of linolenic acid and of antioxidants including vitamin E. Although in our study the linoleic acid (LA)-ω6 to linolenic acid (LNA)-ω3 ratio was kept within the recommended range (<5) (Simopoulos, 2008), the values were higher than those found for animals grazing green pasture (tab. I). The LA to LNA ratios of buffalo's, Friesian and Modicana cows' milk samples (respectively 3.1, 4.5 and 3.8) and of

ewes' milk (2.0) were close to values reported by Soják for TMR-fed cow's and ewes' milk samples (respectively 3.92 vs 2.83), in contrast to lower values referred to pasture-fed cows' and ewes' milk (respectively 1.2 vs 1.8) (Soják et al, 2009).

In addition, it is known that the exposure of ruminants to high environmental temperature determines a vitamin E depleting in answer to increasingly animal's stress status due to changes in diet, to intramammary infections with an increase in milk SCC (Harmon et al, 1997). In fact, in this study the milk samples from all species were characterized by a general SCC increase. In warm months the diet of ruminants is mainly based on concentrates and hay because the lack of green pasture which may lead to changes in rumen fermentation and to the reduction of lipid synthesis in mammary gland (Baldi et al, 2000, Licitra et al, 1998). In table I the results on fat showed a milk fat depression addressable to season effect for all samples. In fact, in period of heat stress the ruminants, to maintain thermal balance, eat less during the day, moreover, the decreased dry matter intake (DMI) with low quality forage and higher levels of carbohydrates contribute to the risk of acidosis with the risk to lower milk fat percentage. In order to prevent acidosis, to the animals should be ensured high energy diets and high quality forages.

4.5   Conclusion

The discrepancies found about the levels of vitamin E in our milk samples might be explained with interspecies variability and the similarity could be the consequence of a combination of feed characteristics.

Although our data on α–tocopherol content in milk samples from different species were within the range of concentrations reported in literature, the values were generally lower. These results might be explained by vitamin E low content in summer time fresh forage and by its probable decrease in answer to a stress status of animals in hot temperature conditions. However, only ewes' milk had the highest content of α–tocopherol. Fat, protein content and SCCs are recognized as indicators of

milk quality that is important on processed milk products. In our study milk samples had high SCCs and low fat values. Data reported in literature on vitamin E content in milk samples from ruminants are lacking in accuracy because they are relative only to α–tocopherol and not to γ-tocopherol. About γ- tocopherol only buffalo's and goat's milk when expressed as mg.L-1 were richer than other milk varieties.

It would be interesting, in future to investigate on γ- tocopherol–relative metabolism in goat and in buffalo, animals already known for their β-carotene- relative metabolism different than other ruminants. Considering the important role attributed to γ isomer in food stability in last decades, other experiments should be carried out for a major knowledge on the role of γ- tocopherol for example during cheesemaking.

In addition, it would be interesting to repeat this study for a whole year to know if season and feed affect γ- isomer content in milk.

In literature, to known beneficial effects of α –tocopherol are reported important roles for γ isomer as a great anti-inflammatory, a major scavenger of reactive nitrogen species deleterious in neurodegenerative diseases, and in superoxide dismutase synthesis. It is reported that the intake of γ tocopherol in Italian population is very low compared to Northern Europe or USA (Gascon-Vila et al, 1999; Wagner et al, 2004), because the main sources of vitamin E in Mediterranean diet such as olive oil are predominant in α – tocopherol. Thus, on the basis that milk and dairy products are widely consumed as food and that for their chemistry are an effective delivery system for vitamin E, might be useful to investigate on new strategies for enhancing in natural way not only α isomer but also the γ isomer levels in milk. Thus, a combined presence of α and γ tocopherol may work in synergy to improve milk oxidative stability but also have positive implications in human health.

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Thanks

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