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TITLE

CLA as a nutraceutical molecule: concentration in foods, estimation of intake in Italy and genetic factors affecting the synthesis in animal tissue.

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"It is not possible for our knowing to go beyond what is strictly necessary for the preservation of life.
The morphology shows us that the senses, the nerves and the brain develop proportionally to the difficulty of feeding."

F.W. NIETZSCHE

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I. BACKGROUND

CLA refers to a group of positional and geometrical isomers of linoleic acid (LA; *cis9,cis12* octadecadienoic acid) with conjugated double bonds. These fatty acids are naturally present in ruminant derived products, thus dairy foods and meat are the main sources. CLA importance is related mainly to the healthy properties of two isomers: *cis9,trans11* (*c9,t11*) and *trans10,cis12* (*t10,c12*) (Bhattacharya et al., 2006; Benjamin and Spener, 2009; Churruca et al., 2009; Stringer et al., 2010; Park et al., 2010; Raff et al., 2009). According to animal and cell culture experiments *c9,t11* plays a protecting role against cancer and atherosclerosis (Bhattacharya et al., 2006), and can also attenuate insulin resistance (Taylor and Zahradka, 2004; Hong et al., 2009).
t10,c12 can be mainly related to the increase in energy expenditure and fat oxidation, decrease of adipocyte size and inhibition of some enzymes of fatty acid metabolism and lipogenesis (Blankson et al., 2000; Bhattacharya et al., 2006).
Moreover Stringer et al. (2010) showed that *t10,c12* isomer prevents hepatic steatosis and higher levels of HDL, improves liver functions and attenuates inflammation. However, these findings are sometimes contrasting regarding the effects *in vivo* in humans.
CLA isomers are synthesized by the ruminal bacteria as intermediates in the biohydrogenation of linoleic acid. *c9,t11* is also produced in tissues and in the mammary gland from the desaturation of vaccenic acid escaped from rumen and it is incorporated in tissue and milk lipids, (Bauman et al. 2001; Griinari and Bauman, 1999). The *c9,t11* isomer covers >90% of the CLA isomers in milk fat, while its proportion in beef fat is only 60-85% (Chin et al., 1992; Shanta et al., 1994; Parodi, 1999).
CLA content in cheese can be affected by several self-linked factors: feeding and starting milk, breeding type, cheese processing and microbial starter. Pasture feeding results in higher levels of CLA than indoor feeding, because of the high LA content of grass (Chilliard, 2001; Delagarde and Peyraud, 2002). Moreover a fibre rich diet is requested for the growth of some bacteria responsible for biohydrogenation such as *B. fibrisolvens*. Then, also the microflora responsible for cheese ripening is affected by

the feeding regimen (Kim et al., 2009). In addition Dihman et al. (2007) indicated breed and stage of lactation as other animal-related factors influencing CLA content. The CLA content in dairy products could be also affected by the processing stages, but results are controversial (Bisig et al., 2007). Anyway it was clearly found that Lactobacilli and other microbial culture could increase CLA content during fermentation (Kim and Liu, 2002) and ripening (Jiang et al.,1998; Sieber et al.,2004).
CLA content in meat can be affected by feeding as in dairy products (Dhiman et al., 1997; Shanta et al., 1994; Stanton et al., 2003); then by muscle type (Rule *et al*., 2002) and by breed and animal age (Dannenberger et al, 2005; Nuernberg, 2005; Padre et al., 2006).
Therefore a strong variability among CLA concentrations in ruminant products can be found.
A needed daily CLA intake could be defined, based on anticancer researches: Ip et al. (1994) reported the lowest effective CLA dose for reducing the cancer incidence extrapolated from animal data as 3g/day. Then Parish et al. (2003) showed that a range between 0.8-3 g/day CLA could be effective. Anyway the data available on human CLA intake are controversial. For example Mele et al. (2013) recently proposed that the evaluation of the CLA needed to exert its properties could not be obtained by the only extrapolation from animal data, but should be instead based on the study of different energy metabolism and other phisiological parameters. Moreover all the surveys conducted on CLA beneficial effects on humans were based on the CLA amount detected in blood, and it is not clear if this data is a single data or if it could cover a long period of time, as for ω3. Moreover there is a lack of studies on the ingestion of CLA, and most of the results on CLA effects on health are extrapolated from animals.
In summary CLA could exert some healthy properties in relation to a needed daily intake, but, at our knowledge, no literature in Italy is currently available on the effective CLA amount in foods and on the relative intake in humans.
Thus a quantification of *c9,t11* and *t10,c12* CLA isomers in foods available in Italy large retail-scale trade was performed, followed by an estimation of the isomers intake in a cohort of the Italian population by a food diary.

The following step of the research project, due to the very low concentration of CLA found in foods, investigated genetic factors affecting CLA in meat, in order to enhance its concentration for final consumers.

Thus the aims of the present work were to estimate the effective CLA intake in Italy through a complete analisys of *c9,t11* and *t10,c12* concentrations in dairy and meat products commonly purchasable, and an evaluation on the genetic factors involved in CLA synthesis in meat.

II. MANUSCRIPT INCLUDED

1. **Dairy food and health**
   Manuscript for "La qualità degli alimenti di origine animale e la salute umana".
   Edited by Associazione per la Scienza e le Produzioni Animali (ASPA). Edizioni Fondazione Iniziative Zooprofilattiche, Brescia.
   In press

2. **Conjugated linoleic acid isomer (cis9,trans11 and trans10,cis12) content in cheeses from Italian large-scale retail trade**
   Manuscript for: *International Dairy Journal*
   Published

3. **Survey on conjugated linoleic acid (CLA) content in monogastric and ruminant meats available in the Italian market**
   Manuscript for: *Italian Journal of Animal Science*
   Submitted to journal

4. **Estimation of c9,t11 and t10,c12 conjugated linoleic acid isomers intake in a cohort of healthy students in Italy**
   Manuscript for: *Public Health and Nutrition*

5. **Breed and dietary linseed and protected fish oil affected gene expression in *longissimus dorsi* muscle of beef**
   Manuscript for: *Lipids*

III. LIPID METABOLISM

Lipids are naturally occurring hydrophobic molecules exerting several biological functions.

They can be divided in saponifiable lipids as triacylglycerols, phosphoacilglycerols, sphingolipids, glicolipids, waxes, and unsaponifiable as terpenes, steroides, prostaglandines and related compounds.

Triacylglycerols in most of human tissues derive from glycerol esterification by three fatty acids (*Fig 1*).

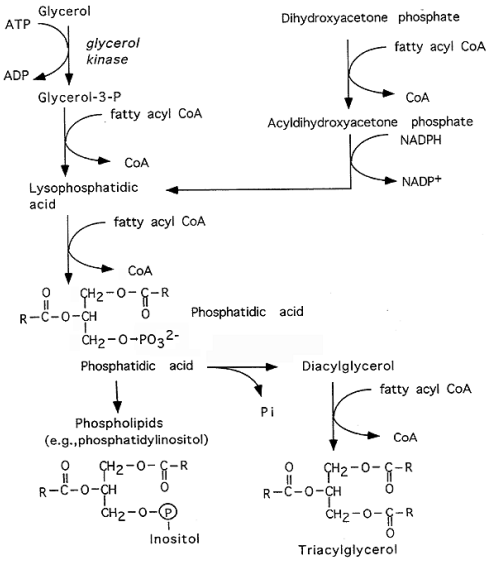

Fig.1. Triacylglycerols Synthesis

The epitelial cells of small intestine produced triacylglycerols from the acylation of digestion derived monoacylglicerols.

Triglycerides stand for the main energy source for our organism.

They could be endogenously reassembled by the lipid digestion and/or they could be synthesized from carbohydrate excess.

Gastric and pancreatic lipase digested lipids in stomach and small intestine producing free fatty acids and monoglycerols. Then they are absorbed in small intestine, assembled to produce triacylglycerols and incorporated in plasmatic lipoproteins (chylomicrons). By lymphatic vessels chylomicrons reach the systemic circulation through thoracic duct in superior vena cava. Then chylomicrons are shrunk in adipose tissue and scheletric muscles by a lipoproteic lipase.

Then free fatty acids can be used, while glycerol is released in blood and is picked up by the liver for glicolisys and gluconeogenesis.

ß-Oxidation

ß-oxidation is the mitocondrial process involved in fatty acids disposal. Free fatty acids are activated as acyl-CoA and relased in cytosol. A carrier is needed for long chain acyl-CoA (12-18 C atoms) to pass through the mitocondrial membrane: the acyl-group is linked to carnitine, due to create acyl-carnitine, a complex able to enter. In mitocondria the acylic group is transferred again to A Coenzime. Short chain fatty acids are instead able to pass the mitocondrial membrane without any carrier and then they are activated in acyl-CoA.

ß-oxidation is summarized below (*Fig. 2*):

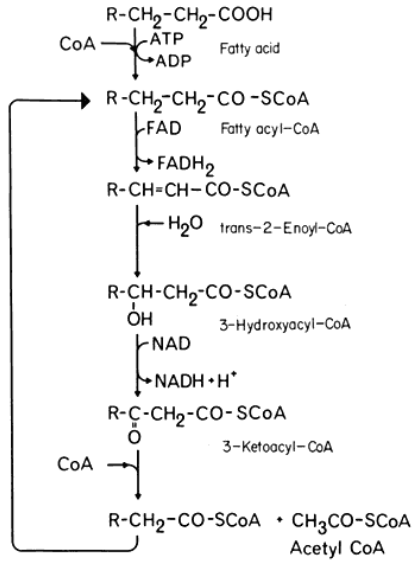

Fig. 2. ß-oxidation

From the estimation of ATP yield in oxidative phosphorilation, each ß-oxidation cycle release:

- 1 Acetyl-Coa (corresponding to 10 ATP in tricarbossilic acid cycle),
- 1 $FADH_2$ (1,5 ATP),
- 1 NADH (2,5 ATP),

producing 14 ATP molecules. However 1 molecule of ATP is spent for fatty acid activation, thus 13 ATP molecules are produced.

Fatty Acid Synthesis

The proces of fatty acid synthesis is outlined below (*Fig. 3*).

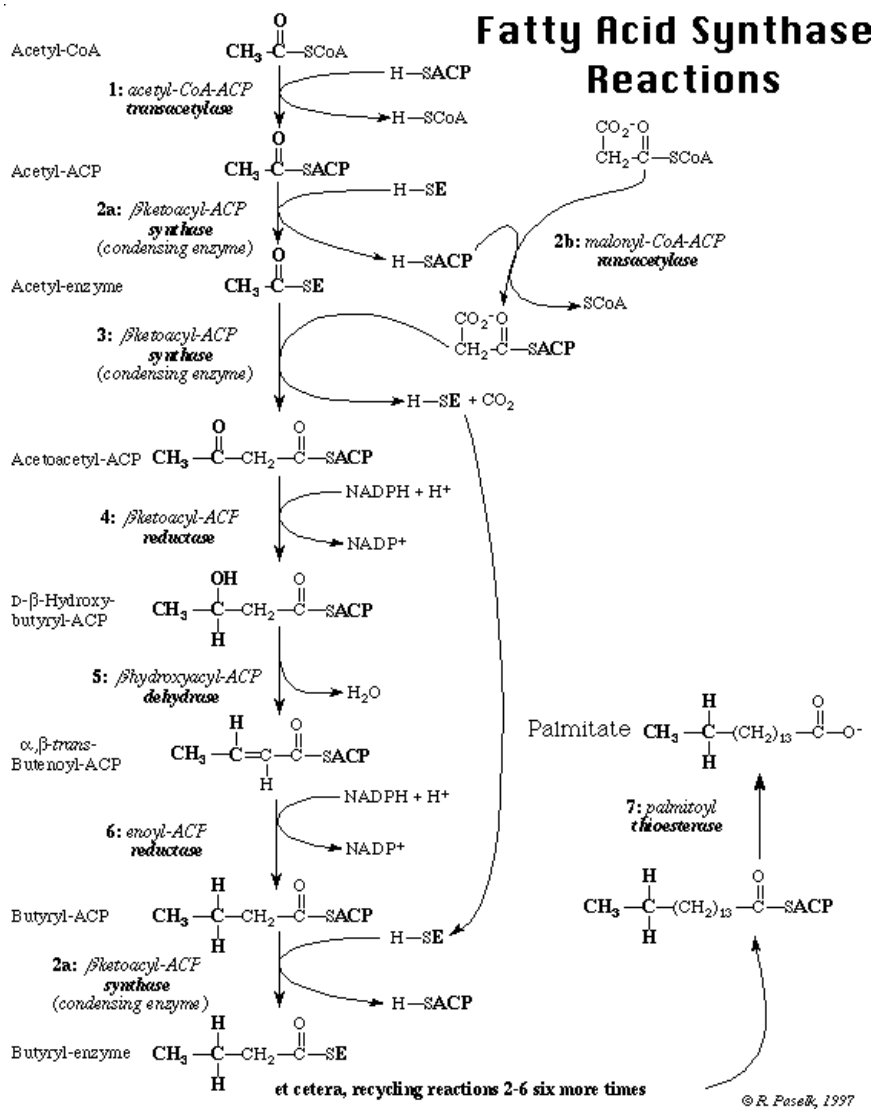

Fig. 3. Fatty Acid Synthesis

Genes Involved

Directly involved in FA synthesis (*Fig. 4*)

ACC (Acetyl-CoA Carboxylase)

FAS (Fatty Acid Synthase)

SCD o Δ^9-Desaturase (Stearoyl-CoA Desaturase)

Δ^6D (Δ^6-Desaturase)

ELOVL5 (Fatty Acid Elongase 5)

Δ^5D (Δ^5-Desaturase)

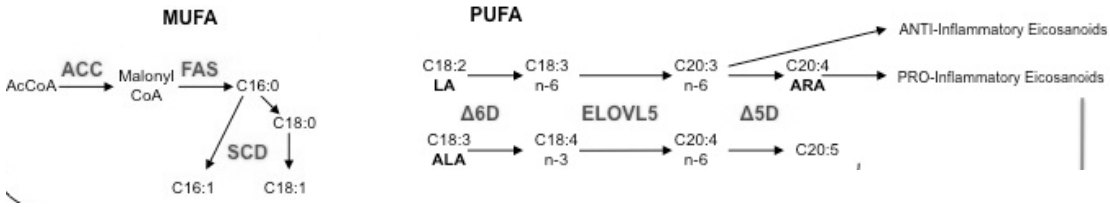

Fig 4. Genes involved in FA synthesis

Involved in lipid and other metabolic pathways related to CLA

AMPKα (AMP-activated Protein Kinase α)---stimulation of hepatic FA oxydation and lipogenesis

PPARγ (Peroxysome Proliferator-Activated Receptor γ)---nuclear receptor required for proper adipose tissue developement and implicated in the regulation of inflammation response

PPARα (*Peroxysome Proliferator-Activated Receptor α)*---nuclear receptor affecting the FA synthesis and the inflammation response

GLUT4 (Glucose Transporter 4)--- glucose uptake, related to insulin resistance

ADIPOQ (adiponectin, C1Q and collagen domain containing)---involved in regulating glucose level and FA oxydation

SREBP-1c (Sterol regulatory element-binding protein 1c)---enhances the transcription of the genes required for fatty acid synthesis and fatty acid elongation including FAS and SCD

ADFP (adipose differentiation-related protein)---related to adypocite differentiation

GPR43 (G-protein coupled receptor 43 or free fatty acid receptor)--- involved in lipid metabolism

STAT5 (signal transducer and activator of transcription 5)---related to cell differentiation and inflammation

IV. CLA BIOSYNTHESIS

CLA Isomers

The acronym CLA refers to positional and geometrical isomers of linoleic acid (LA; *cis9,cis12* octadecadienoic acid) with conjugated double bonds. Among them, *cis*9,*trans*11 and *trans10,cis12* CLA have been recognised as the most biologically active isomers (Bhattacharya et al., 2006).

Due to their origin as intermediates in the biohydrogenation performed by rumen microflora, CLA isomers are naturally present in ruminant derived products.

In milk *c9,t11* CLA is the predominant isomer, as it covers over 90% of all CLAs. (Parodi 1977, Chin et al., 1992) and in beef fat it covers over 75% of the CLA (Chin et al., 1992; McGuire et al., 1999; Yurawecz et al., 1999a).

Sehat et al. (1998) identified the distribution of CLA isomers in cheese fat as *c*9, *t*11 (78 to 84%), *t*7, *c*9 + *t*8, *c*10 (8 to 13%), *t*11, *c*13 (1 to 2%) and *c*12, *t*14. In addition Fritsche et al. (2000) studied the CLA isomers distribution in beef samples and reported again the *c9,t11* CLA as the predominant isomer (72%), followed by *t7,c9* isomer (7.0%).

Despite its biological importance, *t10,c12* isomer in ruminat products covers less than 5% of the total CLA (Khanal and Olson, 2004).

Dhiman et al. (2007) showed 17 natural CLA isomers found in milk, dairy products, beef, human milk, and human adipose tissue using silver ion-high performance liquid chromatography and gas chromatography-electron ionization mass spectrometry: *t12,t14; t11,t13; t10,t12; t9,t11; t8,t10; t7,t9; t7,c9; t6,t8; c12,t14; t11,c13; c11,t13; c10,t12; c9,t11; c8,t10; c7,t9; c9,c11; and c11,c13.*

Synthesis

CLA could be produced both by a ruminal biohydrogenation of C18:2 and C18:3 and by an endogenous synthesis in tissues from a rumen precursor.

Ruminally, CLA is produced as an intermediate during the biohydrogenation of dietary C18:2 and C18:3 to stearic acid (C 18:0).

Endogenously, CLA is synthesized from *trans*11 octadecenoic acid (C18:1, vaccenic acid (TVA)), by the Δ^9- desaturase enzyme. A small portion of CLA produced in the rumen could escape further biohydrogenation to be absorbed by the digestive tract and

then used in tissues. (Griinari and Bauman, 1999).

The endogenous synthesis of CLA from TVA has been proposed as the major pathway of CLA synthesis in lactating cows, accounting for an estimated 78% of the CLA in milk fat (Corl et al., 2001; Dawson et al., 1977)

Rumen origin of CLA

Reiser et al. (1951) and Shorland et al. (1955) firstly demonstrated the hydrogenation of dietary unsaturated fatty acids in rumen.

Griinari and Bauman (1999) described the synthesis of CLA in two main steps.

STEP 1: the Linoleic acid is isomerized to *c9,t11* octadecadienoic acid.

STEP 2: the cis-double bond is hydrogenated, and a trans-monoenoic acid is produced. *trans11* vaccenic acid is accumulated in the rumen, becoming available for absorption in the small intestine, and partially transformed in C18:10 stearic acid.

As a matter of fact CLA and TVA often escape a complete ruminal biohydrogenation, and are absorbed from intestine and incorporated into milk fat (*Fig. 6*) (Griinari and Bauman 1999; Jiang et al., 1996)

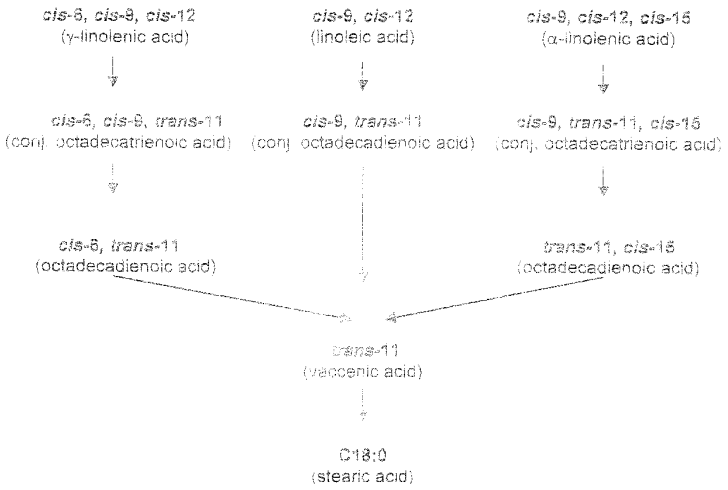

Fig. 6 Predominant pathways of biohydrogenation in the rumen

Similar to the biohydrogenation of C18:2, also C 18:3 fatty acids are are hydrogenated in the same way after isomerization of the cis-12 double bond, ending with the formation of C18:0 in the case of complete biohydrogenation (Harfoot et al 1988; Griinari and Bauman, 1999).

The relatively constant profiles of trans-octadecenoic acids found in ruminant meat and milk fat, where *trans-11* octadecenoic acid is the predominant isomer, suggested a

relatively stable rumen bacteria population. *Butyrivibrio fibrisolvens* is the rumen bacteria mainly deputated to CLA synthesis, operating at the optimal condition of neutral pH (Kim et al., 2000).

Studies with pure strains of ruminal bacteria have shown that most of the bacteria are capable of hydrogenating C18:2 to *t*-C18:1 and related isomers, but only few of them could reduce C 18:2 and C18:1completely to C18:0 (Fellner, et al., 1995).

Interestingly, no single species of rumen bacteria catalyzes the complete biohydrogenation sequence (Harfoot et al., 1988; Kemp et al, 1984).

Griinari et al. (1998) reported that a low fiber diets could affect the trans-octadecenoic acid profile of milk fat, probably due to an altered rumen profile: a predominance of *trans10*-octadecenoic acid among the trans-octadecenoic acid isomer of milk fat was in fact showed.

Therefore a specific *c9,t10* isomerase in rumen bacteria could be involved with the formation of a *t10,c12* conjugated double bond as first intermediate (*Fig.7*)

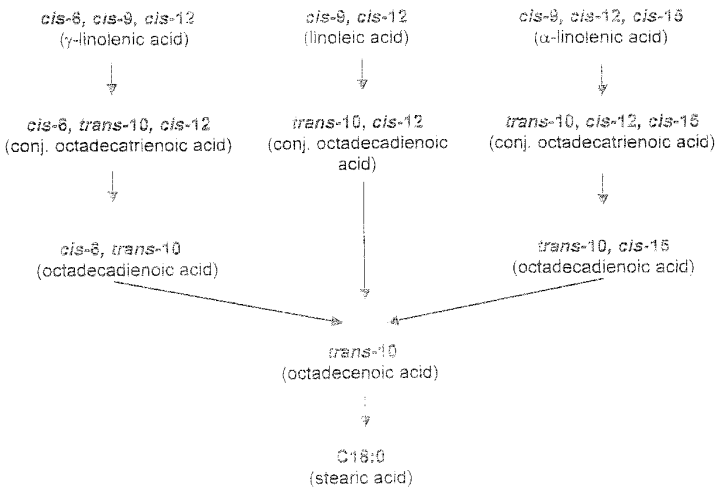

Fig.7. Alternative pathways proposed by Griinari and Bauman (1999)

Thus rumen microflora can vary depending on cows' diet. A diet providing PUFA, in particular LA, has been largely recognised as a factor enhancing CLA amount in milk and meat. Pasture feeding provides a high LA level, and, due to the LA toxicity to rumen microorganisms, biohydrogenation can be considered as a detoxification method. Anyway, Kim et al. (2000) reported that the ruminal biohydrogenation could be activated also by a discrete LA supplementation, avoiding negative effects on the rumen microflora, due to the slowly release of triglycerides with unsaturated fatty acids along ruminal digestion.

Tissue and milk origin

As reported before, the CLA originating from the rumen was found inadequate to account for tissue and milk levels.

Therefore, CLA must also be synthesized in tissues from a precursor of rumen origin (*Fig. 8*). Griinari and Bauman (1999) speculated that *trans-11* octadecenoic acid could be the rumen-derived precursor of CLA in milk, due to the relatively constant ratio of trans-11 octadecenoic acid and CLA found in milk fat across a wide range of diets. They suggested a CLA endogenous production from *trans-11* octadecenoic acid in tissues by Δ^9-desaturase ([a,b]Griinari et al., 1997; Kay et al., 2004).

The endogenous synthesis of CLA by Δ^9-desaturase was largely investigated and the actual estimated endogenous synthesis of *c9, t11* CLA in milk was 64, 78, or 80% of the total *c9, t11* CLA, respectivel from the study of Griinari et al. (2000), Corl et al. (2001) and Lock et al. (2002).

Δ^9-desaturase also showed different and specie-dependent distributions in tissues, and in lactating ruminants the mammary tissue showed the highest activity of this enzyme. (Kinsella et al., 1972).

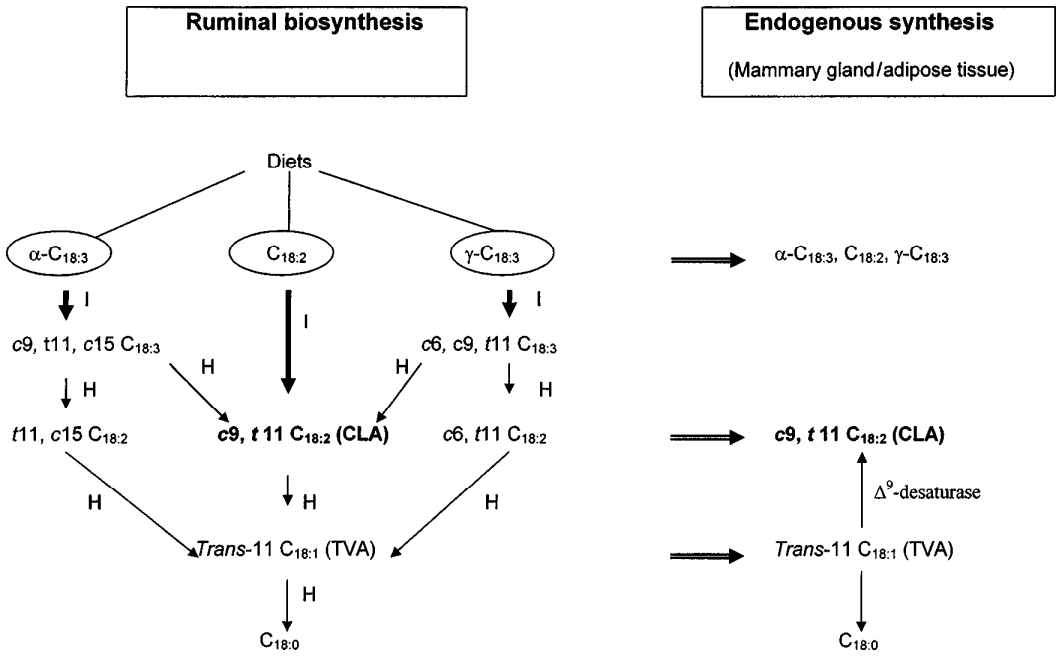

Fig. 8. CLA biosynthesis in rumen and tissues reported by Dhiman et al. (2007)

Human synthesis

An endogenous CLA synthesis in human was suggested from the results of Salminen et al. (1998). The subjects in this study followed a diet basing on dairy products for 5 weeks, then they consumed a diet high in stearic acid or high in vegetable fats (introducing 3g of *trans-11*/day) for a period of 5 weeks. The expected CLA intake based on the diet for the dairy period was 310 mg/d, for the stearic diet 90 mg/d and for the last diet 40 mg/d. The CLA concentrations analysed in serum lipids of the subjects after the different diets were found higher than calculated amount, after the introduction of hydrogenated lipids. Thus the authors concluded that a Δ^9-desaturation of dietary *trans-11* occurred after the consumption of vegetable fat.

V. CLA DIETARY SOURCES

As reported above, the main CLA dietary sources are the dairy and meat products from ruminants. In particular the highest *c9,t11* concentration was found in ewes' milk (Banni et al., 1996; Jahreis et al., 1999) and cheese (Contarini et al., 2009; Cruz-Hernandez et al., 2006; Prandini et al 2011) and in lamb meet (Chin et al., 1992; Fritshe and Steinhart, 1998; Dufey et al., 1999; Wachira et al., 2002; Badiani et al., 2004; Knight et al., 2004).

Dhiman et al. (2007) and Khanal and Olson (2004) reviewed CLA content in foods and showed the total CLA as % of fat as reported in the tables below.

Tab 1. CLA in dairy products (Khanal and Olson, 2004)

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.* (1995)
Cheese	Cheddar	-	0.40-0.47	Lin *et al.* (1995)
Cheese	Swiss	-	0.55	Lin *et al.* (1995)
Yogurt	-	-	0.44	Ma *et al.* (1999)
Yogurt	-	-	0.38	Lin *et al.* (1995)
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.* (1995)
Buttermilk	Cattle	-	0.47	Lin *et al.* (1995)
Evaporated milk	Cattle	-	0.34-0.64	Lin *et al.* (1995)

Tab 2. CLA content in meats (Khanal and Olson, 2004)

Products	Species/breed	Diet	Content	Reference
Beef	Heifers	Concentrate + soy oil	0.34	Beaulieu *et al.* (2002)
Beef	Cattle	Concentrate + extruded soybean	0.73	Madron *et al.* (2002)
Beef	Cattle	All concentrate	0.12	Mir *et al.* (2000)
Beef	Cattle	All pasture finished	1.5	Poulson (2001)
Beef	Cattle	Grass based + concentrate	1.1	French *et al.* (2000)
Ground beef	Cattle	-	0.16	Ma *et al.* (1999)
Ground beef	cooked beef		0.18	Ma *et al.* (1999)
Rib roast	beef	-	0.30	Ma *et al.* (1999)
Rib roast	Cooked beef	-	0.29	Ma *et al.* (1999)
Sirloin	Beef	-	0.12	Ma *et al.* (1999)
Sirloin	Cooked beef	-	0.28	Ma *et al.* (1999)
Beef	Charolais	Concentrate based + linseed	0.80	Enser *et al.* (1999)
Beef	Charolais	Concentrate based + fish oil	0.57	Enser *et al.* (1999)
Beef	Angus × Hereford	Finishing diet + soy oil	0.28	Griswold *et al.* (2003)
Veal	Cattle	-	0.27	Chin *et al.* (1992)
Lamb	Sheep	-	0.35-0.90	Ivan *et al.* (2001)
Adipose tissue	Sheep	Browsed	1.7	Banni *et al.* (1996)
Lamb	Sheep	-	0.06-0.31	Mir *et al.* (2000)
Lamb	Sheep	Browsing	1.5	Fogerty *et al.* (1988)
Lamb	Sheep	Beet pulp + safflower	0.65-0.98	Bolte *et al.* (2002)
Lamb	Sheep	-	0.56	Chin *et al.* (1992)
Ground turkey	Turkey	-	0.25	Chin *et al.* (1993)
Chicken	Chicken	-	0.09-0.2	Chin *et al.* (1992)
Pork	Swine	-	0.12	Chin *et al.* (1993)
I/M1 fat	Swine	2.5% CLA in diet	1.0	Joo *et al.* (2002)
S/C2 fat	Swine	1.0% CLA in diet	2.16	Thiel-Cooper *et al.* (2001)
Lean tissue	Swine	1.0% CLA in diet	0.37	Thiel-Cooper *et al* (2001)
S/C2 fat	Swine	1.0% CLA in diet	4.0	Ramsay *et al.* (2001)
Back fat tissue	Swine	2.0% CLA in diet	2.0	Bee (2000b)
Omental fat	Swine	2.0% CLA in diet	2.2	Bee (2000b)
L. dorsi	Swine	2.0% CLA in diet	0.98	Bee (2000b)
Breast muscle	Broiler	1.0% CLA in diet	5.2	Szymczyk *et al.* (2001)
Belly fat	Swine	1% CLA oil in diet	0.76	Eggart *et al.* (2001)
L. muscle	Swine	1% CLA oil in diet	0.28	Eggart *et al.* (2001)
Egg yolk	Chicken	1.0% CLA in diet	0.30	Jones *et al.* (2000)
Egg yolk	Chicken	1.0% CLA in diet	1.4-3.2	Raes *et al.* (2002)
Egg yolk	Chicken	Concentrate	ND[3]	Raes *et al.* (2002)
Egg yolk	Chicken	Concentrate	ND[3]	Yang *et al.* (2002)

[1]Intramuscular. [2]Subcutaneous. [3]Not detected.

Samples*	Total CLA** (% of fat)	*c*9, *t*11 CLA***(%)
Fluid milk products		
Whole milk[60,86,98,99,108,114,118,191,202,211]	0.34–0.68	82–97
Evaporated milk[211]	0.49	—
UHT milk[199]	0.80	—
Homogenized milk[1]	0.55	92
Condensed milk[1,199]	0.63–0.70	82
Cultured buttermilk[1,191]	0.54–0.67	89
Cheeses		
Cheddar[52,193,197,198,202,211]	0.40–0.53	78–82
Feta[52]	0.49	81
Cottage[1,202,211]	0.45–0.59	83
Mozzarella[1,52,86,193,202,211]	0.34–0.50	78–95
Processed cheese[196,197,202,211]	0.41–1.07	75
Processed American[1,52,211]	0.36–0.50	79–93
Processed Cheddar[52]	0.50	84
Processed Parmesan[202]	0.53	—
Fermented dairy products		
Plain yogurt[1,193,194,199,200,202,211]	0.38–0.88	83–84
Lowfat yogurt[1]	0.44	86
Butter[1,193,199,202]	0.47–0.94	78–88
Sour cream[1,193,202]	0.46–0.75	78–90
Ice cream[1,193]	0.36–0.50	76–86

Samples*	Total CLA** (% of fat)	*c*9, *t*11 CLA***(%)
Ruminants		
Beef		
Ground[1,61,192,202,212]	0.16–0.43	72–85
Round[1,56]	0.29–0.68	57–79
Ribeye[56,202]	0.30–0.64	61
T-bone[56]	0.61	59
Sirloin[56,202]	0.12–0.58	59
Frank[1]	0.33	83
Smoked sausage[1]	0.38	84
Veal[1]	0.27	84
Lamb[1,58,177,180,181,199]	0.18–1.20	92
Non-ruminants		
Turkey[1,58,199]	0.20–0.25	40–76
Turkey frank[1]	0.16	70
Smoked turkey[1]	0.24	62
Pork[1,58]	0.06–0.13	25–82
Smoked bacon[1]	0.17	76
Chicken[1,58,199]	0.09–0.15	67–84
Rabbit[58]	0.11	27

Table 3 and 4. CLA in dairy and meat products reported by Dhiman et al. (2007)

As expected, very small amounts of CLA have been found in milk, meat, or egg from non-ruminants (Bee, 2000; Chin *et al*., 1992, 1993). In tissue fat of chicken or swine fed normal concentrate diets, the CLA content was only around 0.1% of the total reported fat (Chin *et al*., 1993). However, feeding CLA as low as 1% of the diet may boost its content in the food products of non-ruminant origin by several folds (Raes *et al*., 2002; Ramsay *et al*., 2001; Thiel-Cooper *et al*., 2001).

The CLA content in human milk was reported around 0.1% (Park *et al*., 1999).

CLA concentration in foods was studied through different countries, and due to the different environment and feeding systems, some variations were found (Fogerty et al., 1988; Shanta et al., 1997). Australian beef showed the highest values, with CLA as 1% of total fatty acids; CLA concentrations in german foods represented 0.65% of total fat; US beef hade the lowest values and CLA ranged from 0.3 to 0.5% of total fat. Moreover Wood (1983) found that US beef fat contained trans 10 as the major octadecenoic acid isomer rather than t11 as in fat from European beef (Wolff, 1995).

In conclusion dairy products and meat could be CLA sources, but in each country a study must be performed, due to the variations that occurrs depending on animal diet, enviroment and dietary habits.

VI. FACTORS AFFECTING CLA

The fatty acid compositions and consequently the CLA concentration of animal tissues can be strongly affected by numerous cross-linked factors: nutritional status, depot, breed, feeding and lactation stage and dietary factors (Duckett et al., 2002; Aharoni et al., 1995; Dihman et al., 1996; Kelly et al., 1998; White et al., 2001). Moreover an individual variation in milk CLA concentration was reported (Peterson et al., 2002; Kelsey et al., 2003). Therefore CLA content in ruminant products can be affected by all the factors above mentioned, and other factors related to the product manufacturing, as cheese processing parameters: aeration (Ha et al., 1989), temperature (Shanta et al., 1992), milling pH, additives, and ripening (Lin et al., 1995) and the microbial cultures used.

6.1 Diet

As reported before, CLA in ruminant tissue and milk derived from the isomerization of LA and LNA during the biohydrogenation by rumen bacteria. The lipid availability and the microflora are key factors in the synthesis of CLA and they can be modulated by the source and the quantity of lipid substrates available (Chouinard et al., 2001; [a]Kim et al., 2003) and by the feeding regimen.

Griinari and Bauman (1999) divided the CLA dietary affecting factors in three groups: 1) providing lipid substrate for CLA or *trans-11* octadecenoic acid synthesis in the rumen; 2) affecting the rumen microflora involved in the biohydrogenation of fatty acids; 3) involving both lipid substrate and bacterial populations.

Useful lipid substrates for rumen biohydrogenation could derive mainly from sunflower and linseed oil: sunflower oil is a source of C18:2 linoleic acid (LA), while linseed oil supplies C18:3 linolenic acid (LNA).

Kelly *et al*. (1998) reported that sunflower oil resulted in highest CLA concentration in milk fat if compared with other oil supplementations (peanut oil, sunflower oil, and linseed oil, which are high in OLA, LA, and LNA, respectively). Similarly Dhiman et al. ([a,b]1999, 2000) studyied the effect of diet on CLA content of cows' milk and found that some high-fat diets (mainly from soybean) could increase milk CLA content. However it must be underlined that a too high fat intake could depress milk fat

synthesis and in that case could affect the final CLA amount produced.
The second group of dietary factors is instead related to a shift in the rumen microflora due to the effects of the ratio on the pH of the rumen.
In particular a low-fiber diet was found to reduce the ruminal pH, increasing *t10,12* in rumen digesta and in milk fat (Griinari and Bauman, 1999). Based on studies with lactating cows, Griinari et al. (1998) reported that this type of diet would lead to a change in the rumen environment, and finally would result in a decreased formation of *t-11* octadecenoic acid in the rumen and, as a consequence, in a decreased CLA concentration in tissues.
Thus the lower relative proportion of the *c9,t11* isomer related to a a change in the ruminal biohydrogenation could be induced by traditional high concentrate/low fiber finishing diets, and an altered profile of CLA isomers characterized by an elevated level of *t10,c12* isomer could be found in the milk fat of lactating dairy cows fed a low fiber diet.
Moreover it was reported that also feeding seeds from linseed or soybean in a low forage diet regimen (30:70 forage to concentrate ratio) would not increase milk fat CLA (Chilliard *et al*., 2003). It was hypothesized that low forage in the diet could reduce the rumen pH below 6.0, which has negative effects on both CLA and TVA concentrations in the rumen (Troegeler-Meynadir *et al*., 2003; Martin and Jenkins, 2002).
The effect of dietary fiber on CLA content in meat of growing cattle was demonstrated in a study in which pasture feeding increased CLA content of body fat compared with that observed with traditional grain diets (Shanta et al., 1997).
Moreover the CLA enhancing role of diet, in relation to a balance of the rumen environment, was demonstrated also by Fritshe and Fritshe (1998). The authors analyzed subcutaneous and intermuscolar fat samples from cattles after a corn-silage feeding providing a sufficient amount of fiber to maintain normal rumen fermentation, and found that *c9,t11* isomer covered >90% of total CLA.
Regarding the group 3, a lot of studies confirmed the role of pasture feeding in enhancing the milk CLA concentrations in dairy cows with an increase in CLA related to increasing proportions of pasture in the diet (Jahries et al., 1997; Stanton *et al*., 1997; Kelly *et al*., 1998; [a]Dhiman *et al*., 1999; Collomb et al., 2002; Collomb et al., 2004; Leiber et al., 2005; Parodi et al., 1999; Rego et al., 2005). For example Couvreur *et al*. (2006) found a linear increase in CLA, MUFA and PUFA in milk at the expense

of SFA percentage replacing corn silage with increasing proportions of fresh cut grass in the cow diet.

The enhancing effect on CLA amount of pasture feeding is mainly due to the supplementation of PUFA, precursors of CLA. Linoleic acid is basic for the rumen synthesis of TVA and its subsequent desaturation to *c*9,*t11* CLA in the mammary gland (Bauman *et al*., 2003).

In contrast the ensilaging includes conservation processes thet may reduce where PUFA in herbage: Elgersma *et al*. (2003) reported a decrease in the proportion of LNA in ensiled material in comparison to fresh grass.

Moreover the hay fed during summer could have higher content of CLA precursors than hay fed in winter-early spring, due to the lower oxidation process related to the duration of the storage (Dewhurst *et al*., 2006).

As a matter of fact the CLA enhancing role of fresh grass is more evident during summer: Prandini et al. (2009) showed that Grana Padano produced in lowland- hill in Italy in summer months, after a greater quantity of fresh forage, contained higher amount of CLA, oleic acid and MUFA and lower levels of SFA if compared to spring cheese.

Ledoux et al. (2005) found important differences in CLA content between summer and winter butter (0.80 and 0.45 g CLA·100 g^{-1} butter, respectively). The same authors showed that milk from mountainous areas had a higher CLA content than milk from lower regions. In their recent review on the variation of CLA in unprocessed milk fat, Collomb et al. (2006) reported values ranging from 0.2 to 5.37 g·100 g^{-1} fat. However the highest reported value was from a study of Shingfield et al. (2006) obtained by a diet supplemented with fish oil and sunflower oil.

6.2 Lactating season

The lactation season has been thought to have a strong impact on cheese quality. Since 1953, McDowell and McDowell showed a seasonal variation of CLA, found substantially higher in spring and summer (when cows were pastured) than in fall and winter (when cows were house-fed), that could be compared with the trend of oleic acid content in butter fat. Then Parodi et al (1999) reported from literature the same trend of CLA amount in milk during seasons and Rego et al. (2008) again noted that

summer milk fat contained more conjugated dienoic fatty acids than winter milk fat. Moreover differences of fatty acid composition and CLA concentration in cheese were seen in the work of Prandini et al. (2009) between spring and summer. In this work summer Grana Padano from mountains (M-GP) had a better fatty acid composition than spring M-GP, with higher levels of beneficial fatty acids for human health (CLA, TVA, oleic acid, MUFA and PUFA) and lower levels of detrimental fatty acids (SFA). Thus, from all of these results, the effect of the production season of the milk used in cheese manufacturing and of the relations between production season and area could be clearly underlined.

6.3 Breed

Kelsey *et al*. (2003) showed that Holstein could provide a greater CLA content of milk compared to Brown Swiss in similar feeding condition; however, despite the significance of their results, the differences were inconsequential if compared with the effects of dietary manipulation or the variation among individuals. Thus they concluded that breed effect could account for only □0.1% of the total variation in the CLA concentration in milk fat.

Thus more studies on the genetic mechanism involved in CLA pathways must be performed to understand the breed effect. In this work a short trial was conducted to underline differences in CLA synthesis by two breeds drammatically different in the lipid metabolism (See Manuscript 5).

6.4 Microbial starter

A lot of studies were carried out regarding the influence of microorganisms on CLA content in dairy products and a review on these studies was published by Sieber et al. (2004). Strains of lactobacilli, bifidobacteria and propionibacteria were found to be able to convert linoleic acid efficiently into CLA in milk. However, the same effects were not showed in the investigations on yoghurt and cheese.

Bifidobacteria, propionibacteria, *Lb. plantarum*, *Lb. rhamnosus* and *Lb. acidophilus* mainly showed a high linoleic acid conversion.

From literature, free linoleic acid has been recognized as a needed substrate, even if a careful check must be done regarding its optimal amount, because a too high amount

of linoleic acid could reduce the conversion rate into CLA (Alonso et al, 2003; Ogawa et al, 2005; Oh et al., 2003).

Bifidobacteria

Coakley et al. (2003) reported the production of *c9,t11* CLA isomer by some propionibacteria strains from free linoleic acid. Some *B. breve* and *B. dentium* strains were reported as the most efficient CLA producers. For example *B. breve NCFB 2258* converted 65% of the linoleic acid into CLA.
The CLA production by bifidobacteria was suggested by the authors as a possible mechanism for health- enhancing properties, in according to Oh et al. (2003), who screened about 300 colonies of bifidobacteria strains isolated from breast-fed infants regarding their ability in producing CLA. Reasonable amounts of CLA were produced by different colonies; among them *B. breve* and *B. pseudocatenulatum* were the most active: the total CLA conversion from 0.01% linoleic acid was 78% for *B. breve* and 69% for *B. pseudocatenulatum.*

Propionibacteria

Jiang et al. (1998), Rainio et al. (2002), Ross et al. (2000) and Sieber 2004 reported the efficiency of propionibacteria in the conversion of linoleic acid into CLA with rates of up to 87%. In the work of Jiang et al. (1998) strains of *Propionibacterium freudenreichii* ssp. *freudenreichii* and *P. freudenreichii* ssp. *shermanii* converted free linoleic acid into CLA (mainly *c9,t11*) in skimmed milk. Also Xu et al. (2004) found a CLA increase due to some strains of *Propionibacterium freudenreichii* ssp. *shermanii* and *P. freudenreichii* ssp. *freudenreichii*.
Anyway Gnädig et al. (2004), investigating the effect of some strains of *Propionibacterium freudenreichii* in comparison with cheese manufactured without propionibacteria, did not reported any alteration in CLA content or CLA isomer composition.

Lactobacilli

A lot of study on the Lactobacilli effect on CLA concentration were conducted (Jiang et al. (1998), Lin et al. (1999), Pariza and Yang (1999, 2000), Ogawa et al. (2001),

Kim and Liu (2002), Kishino et al. (2002), Coakley et al. (2003) and Alonso et al (2003)). The results were contrastant: Kim and Liu (2002) found an increasing CLA content in whole milk due to the lactobacilli fermentation, while Coackley et al. (2003) did not reported any detectable CLA production by lactobacilli, lactococci and pediococci.

6.5 Cheese processing

Contrasting results were found regarding the existence of cheese processing effects on CLA concentration.

Steinhart (1996) reported that processing, such as heating, could change the CLA isomer distribution in dairy products while the total CLA content remained unchanged after conventional processing.

Ha et al. (1989) and Garcia-Lopez et al. (1994) reported increased levels of CLA in processed cheeses as compared with natural cheeses.

In contrast Bisig et al. (2007) reported that manufacturing processes have no effect on CLA, while Kim et al. (2009) reported that CLA content could be affected by fermentation, temperature, pH, additives and ripening. Ha et al. (1989) and Shanta et al. (1992) reported the importance of aeration, heating temperature and pH, because LA isomerization is a pH-dependent reaction. Finally Kim and Liu (2002) reported that lactic acid bacteria increased the CLA content during cheese fermentation and an increase in CLA content by microbial cultures during cheese ripening was found also in the works of Jiang et al. (1998) and Sieber et al. (2004).

Kim et al. (2009) reported that both in pasture and indoor feeding the extension of ripening period increased CLA content in cheese: CLA in processed cheese was enhanced by ripening for 4 months compared with that in raw milk samples. However, CLA level in 7 month-aged cheese did not show a significant difference between pasture and indoor feeding. This suggested that the CLA level in milk from indoor feeding could be strogly affected than the one from pasture feeding by the long-term ripening process. Also Lin et al. (1999) and Gürsoy et al. (2003) detected the highest CLA content in cheeses with a long aging time, respectively analyzing Cheddar and Turkish cheeses. In addition Ha et al. (1989) and Prandini et al. (2001) reported higher CLA contents in cheese than in the raw milk.

Anyway Werner et al. (1992), Jiang et al. (1997), Gnädig et al. (2004), Nudda et al. (2005) and Ryhänen et al. (2005) did not observed any process effect on the CLA content in Edam cheese, Swedish Swiss- type cheese, French Emmental and other hard cheeses, and Pecorino Romano cheese and Ricotta cheese.

The heating temperature during cheese processing could also affect CLA concentration in processed cheese: Cheddar cheese heated to 80-90 □C had a higher CLA content if compared with cheese heated to 70 □C (Shanta et al, 1992).

Anyway, the heating was proposed as the only process involved in CLA increase during cheese manufacturing by Fernandez-Garcia et al. (1994) that suggested that high temperature enhanced the formation of LA radicals resulting in a conjugated system in the fatty acid backbone.

These findings were discordant with the studies of van Nieuwenhove et al. (2004) and Luna et al. (2004) who did not found any increase in CLA levels in imlk fat after heating at a high temperature, and Gnädig et al. (2004), studiyng mildly heating processes on milk (68 °C/20 s) and several cooking/moulding temperatures of 52 °C/50 °C or 48 °C/48 °C or 50 °C/ 50°C, did not reported any effect on CLA content in French Emmental cheese.

In addition Campbell et al. (2003) and Precht et al. (1999) observed losses of CLA through high- temperature-short-time pasteurisation or more severe heat treatment up to 200 °C.

As previously reported, also fermentation could be another process involved in enhancing the CLA concentration in dairy product. The activity of some lactic acid bacteria increased CLA content in the study of Kim an Liu (2002).

In addition, the role of pH must be reminded. As reported in paragraph 6.1, LA isomerization is a pH dependent enzymatic reaction, thus an acidic environment induced by high concentrate could be antagonistic to CLA production. Thus another variable that must be monitored during the cheese manufacturing is pH.

VII. CLA HEALTHY PROPERTIES

CLA isomers were found to exert healthy effects with anticarcinogetic (Ha et al., 1990; Ha et al., 1987; Ip et al., 1991; Liew et al., 1995; Belury 2002; Belury et al., 1996; [b]Ip et al, 1999), anti-atherosclerotic (Lee et al., 1994; Nicolosi et al., 1997; [a,b]Kritchevsky et al., 2000; Koba et al., 2002), anti-insulin resistance (Ryder et al., 2001; Houseknecht et al., 1998), anti-inflammatory (Yang et al., 2003; Yu et al., 2002; Iwakiri et al., 2002; Miller et al., 1994; Cook et al., 1993) and anti-adipogenic properties (Park et al., 1997; Park et al., 1999; Pariza et al., 2001; Luna et al., 2004; Luna et al., 2005; Lynch et al., 2005).

Some studies reported CLA positive physiological effects using mixtures mostly of *c9,t11* and *t10,c12* CLA in approximately equal amounts and very low amounts of the others isomers, while other studies showed separate actions of *c9,t11* and *t10,c12* isomers. Thus it could be suggested that some effect are isomer-dependent and others others are induced and/or enhanced by the synergic action of the two isomers.

7.1 Anti-carcinogenetic effect

CLA has been described as an anti-carcinogenic agent since the last two decades, due to its role in inhibiting the chemically-induced carcinogenesis in several rodent models (Ha et al., 1990; Ip et al., 1991; Liew et al., 1995). Based on diet and cancer risk studies, and on CLA amount required for an anti-carcinogenic response extrapolated from rats to humans, Ip et al. (1994) proposed a needed daily CLA intake ranging from 55 mg above basal CLA intake to 3.0 to 3.5 g/d to provide anti-carcinogenic response in humans.

A good skill in the prevention of carcinogenesis was showed by CLA in different tissues (Dilzer *et al.*, 2012) and in a large number of animal studies (Lee et al., 2005; Bhattacharya et al., 2006; Kelley et al., 2007).

However the efficacy was reported mainly in *in vitro* and animal studies, while only few human studies were conducted and controversial results were reported.

CLA could be involved in cancer prevention modulating eicosanoids production, interfering in cell signaling pathways, inhibiting DNA synthesis, promoting apoptosis, and modulating angiogenesis (Masso-Welch et al., 2004; Lee et al., 2005; Lee et al.,

2006; Wang et al., 2005; Beppu et al., 2006; Bhattacharya et al., 2006; Kelley et al., 2007; Flowers and Thompson, 2009; Hsu et al., 2010).
The first human study was conducted by Knekt et al. (1996).

In this study the dairy intake in 4697 cancer free women was analyzed; then food consumption data from these subjects were collected for the following 25 years. The results showed a significant inverse gradient between milk consumption and breast cancer incidence within participants. This suggested a protective effect linked to milk consumption and CLA was suggested as one of the potential active components.
Moreover Aro et al. (2000) conducted an analysis on dietary habits in a cohort of Finnish patients with breast cancer, and found that a diet consisting of CLA-rich foods, especially cheese, may have anticarcinogenic effects with regard to breast cancer in post- menopausal women. However, the study design could not allow the assessing of independent effects of CLA. In addition, in other works, no effect or only a weak positive correlation was showed between CLA intake and breast cancer incidence (Voorrips et al., 2002; McCann et al., 2004).
Dietary CLA intake and CLA levels in breast adipose tissue at the time of diagnosis or subsequent the development of metastasis did not show any significant association between CLA levels and prognostic factors or risk of metastasis or death (Chajes et al., 2002-2003). Similarly, no correlations between serum CLA levels and breast cancer incidence were found by Rissanen et al. (2003).
Larsson et al. (2005), reported instead an inverse correlation between CLA and colorectal cancer incidence in women.
Thus the lack in sufficient positive results do not allow to demonstrate the CLA efficacy against cancer in humans. More trials are necessary in order to determine the mechanisms of action and the specific effects of CLA isomers associated with cancer.

Gastrointestinal and colon cancer

Information on the beneficial effects of CLA on gastrointestinal and colon cancer has been derived mainly from animal and in vitro studies. In the first decade of 2000 a lot of studies were conducted about this issue and quite all of them agreed in the acknowledgment of a beneficial role of CLA isomers in preventing this type of cancer (Bhattacharya et al., 2006; Wahle et al., 2004).

It was showed by [a]Cho et al. (2003) that CLA could inhibit cell proliferation and induce apoptosis of HT-29 cells. Another study suggested that *t10,c12* (not *c9,t11*) inhibited Caco2 colon cancer cells through decreased IGF-II secretion ([a]Kim et al., 2002). As a matter of fact the same group reported that CLA-associated benefits could be in part associated with its skills in decreasing the insulin-like growth factor (IGF) II synthesis and in down-regulating the extracellular signal-regulated kinase-1/2 pathway and IGF-I receptor signaling ([b]Kim et al., 2003). In the same year [b]Cho et al. (2003) obtained similar results in HT-29 cancer cells where *t10,c12* decreased viable cell numbers dose dependently, concluding again that the inhibition of H-29 cells by *t10,c12* isomer was mediated through the inhibition of IGF-II secretion.
In 2006 Lee et al. showed that *t10,c12* isomer repressed cell proliferation and induced apoptosis.
Moreover the usual contemporary presence of the two isomers must be considered: individual isomers were found to have stronger inhibitory effects if compared to a mixture of CLA isomers in a study evaluating the effects of 98% pure *c9t,11* and *t10,c12* CLA isomers in benzo[a]pyrene-induced forestomach neoplasia (Chen et al., 2003). Anyway a recent work of Zhong et al. (2012) reported that the *c9,t11*-CLA and *t9,t11*-CLA mixture (1:1 v/v) could inhibit the growth of Caco-2 cells in a dose and time dependent manner, and can induce the apoptosis of Caco- 2 cells.
Some studies investigated the dose needed to exert these beneficial effects: Park H,S. et al. (2001) showed that 1% CLA of the diet for 30 weeks reduced 1,2-dimethylhydrazine (DMH)-induced tumor incidence in the colon of SD rats; Cheng et al. (2003) examined the dose-dependent inhibitory effects of CLA on mammary and colon carcinogenesis induced by treatment with 7,12-dimethylbenzanthracene and DMH in SD rats and found again the dietary level of 1% CLA as the optimal dose for suppression of carcinogenesis in both target organs.
Anyway controversial data were found in a study on Min mouse model of intestinal carcinogenesis where animals were fed purified isomers at 1% of the diet: the *t10,c12* isomer was found promoting carcinogenesis (Rajakangas Jet al., 2003).
In 2005 a study in BALB/c nu/nu mice, inoculated with MKN28 (human gastric cancer cells) and Colo320 (human colon cancer cells) in their peritoneal cavity, showed decreased metastatic foci in peritoneal cavity with CLA intake, indicating that CLA inhibited metastasis of human gastric and colon cancer cells (Kuniyasu et al., 2005).
Anyway, as reported before, there is unfortunately a lack of human studies.

Larsson et al. (2005) reported a decrease of 13% in the risk of colorectal cancer and of 34% of distal colon cancer for a daily increment of two servings of high-fat dairy foods. However the authors concluded that the observed protective effect of high-fat dairy foods could only be partly attributed to CLA intake.
Thus in vivo and in vitro studies suggested that both CLA isomers in equal proportion either the *t10,c12* isomer alone could prevent gastro-intestinal and colon cancer. However, more human studies are needed to propose CLA isomers as an active agent in preventing cancerogenesis.

Breast cancer

Initial in vitro study in MCF-7 breast cancer cells showed that CLA was growth-inhibitory in culture, but was more cytotoxic to MCF-7 cells (Shultz et al., 1992). Interestingly, CLA inhibited growth and thymidine incorporation of MCF-7 cells, whereas LA was found to be stimulatory (Cunningham et al., 1997). The effect of CLA could be mediated through lipoxygenase inhibition because the CLA addition with a lipoxygenase inhibitor resulted in synergistic growth suppression.
Moreover Miller et al. (2001) reported that the growth- suppressing effects of CLA isomers in MCF-7 cells might be related to changes in arachidonic acid distribution and to an altered PG profile.
Different mechanisms were hypotesized for *c9,t11* and *t10,c12* isomers in the inhibition of MCF-7 cells proliferation.
Chujo et al (2003) showed that *t10,c12* inhibited cell proliferation when induced by insulin and estrogen. None of these factors instead affected *c9,t11*-mediated inhibition of cell proliferation.
Moreover in 2004 CLA isomers were showed to down-regulate the estrogen receptor expression at mRNA and protein levels, and to decrease binding of nuclear protein to a normal estrogen response element. Thus in this study the antitumoral activity could be explained by antiestrogenic properties (Tanmahasamut et al., 2004).
Wang et al. (2005) found again that both the isomers seemed to be active: MCF-7 cancer cells were co-cultured with human breast stromal cells in the presence of *c9,t11* and *t10,c12* CLA isomers and both decreased mRNA vascular endothelial growth factor (VEGF) expression and protein levels in the cancer cells (Wang et al., 2005). However, *t10,c12* CLA appeared to be the more active isomer of the two.

Also in animal studies CLA was found to be an effective agent in inhibiting the development of mammary tumors. In one of the first studies, Ip et al (1991) fed rats with diet supplemented with 0.5%, 1%, or 1.5% CLA from 2 weeks before the carcinogen administration and continued until the end of the experiment. The mammary adenocarcinomas decreased by as much as 60%; the final tumor incidence and cumulative tumor weight were lowered in rats fed with CLA diets.
Another study by Ip et al. (1996) reported that the protective effect of CLA was not influenced by the level or type of fat of the diet. More recent studies by Ip et al. (Ie. Ip et al., 2003) indicated that CLA may prevent breast cancer also through its antiangiogenic activity.
Some studies coupled the CLA action with known anticarcinogenic agents. A work of Tao et al. (2012) showed an enhanced anticancer activity of the Gemcitabine if coupled with CLA.
Only few clinical studies were conducted to study the effects of CLA on breast cancer and no correlations were found.
Thus, although results from in vitro and animal studies reported a cancer inhibition related to a CLA introduction, no demonstrations nowadays exsist on CLA role against human breast cancer.

Prostate cancer

In vitro studies suggested that both the CLA isomers *c9,t11* and *t10,c12* could have beneficial effects against prostate cancer.
Palombo et al. (2002) and Ochoa et al. (2004) analyzed the effects of CLA isomers on PC-3 prostate carcinoma cell line in vitro. The isomers differed in their antiproliferative activity and *t10,c12* was found more beneficial comparing with *c9,t11*. The second study concluded that the effects of *t10,c12* were mediated through modulation of apoptosis and cell cycle control, while *c9,t11* mediated its effects through alternation in AA metabolism.
A study conducted on LNCaP prostate cells reported that the antiproliferative activity of CLA isomers and their role in affecting the protein kinase C isoforms could partly explain their antitumorogenic activity (Song et al. 2004).
The lack in in vivo and human experiments could not allow any conclusion regarding the beneficial effects of CLA on prostate cancer.

Angiogenesis

Masso-Welch et al. (2002) and Ip et al. (2003) showed a CLA-dependent inhibition of angiogenesis in vivo in implanted rat breast tumors. CD2/F(1) mice were given angiogenic challenge after treating them with 1% and 2% CLA for 6 weeks.
CLA-fed mice had lower serum and mammary gland levels of VEGF. In these studies, *c9t11* and *t10c12* CLA isomers inhibited angiogenesis in vitro dose dependently. Thus it was suggested that the anti-angiogenic effects of CLA could be mediated, in part, through the VEGF and its Flk-1 receptor inhibition.
Moreover another study by Masso-Welch et al. (2004) showed that both *c9,t11* and *t10,c12* CLA at 0.5% and 1.0% of the diet could inhibit angiogenesis in vivo and decrease VEGF in CD2/F(1) mice. However the proangiogenic hormone leptin, was decreased only with *t10,c12* CLA diet.
Another factor involved in angiogenesis is the basic fibroblast growth factor (bFGF). Moon et al. (2003) showed a CLA inhibition of bFGF-induced angiogenesis in vivo and decreased bFGF-induced endothelial cell proliferation and DNA synthesis in vitro. Thus the results suggested a positive effect of CLA isomers against angiogenesis, and that the anti-angiogenic activity could be the CLA mechanism to inhibit cancers. However no published reports evaluating the effects of CLA on angiogenesis in humans are currently available.

7.2 Anti-atherosclerotic effects

In the work of Lee et al. (1994) a diet containing a CLA isomer mixture (0.5 g/day) for 22 weeks determined a lower atherosclerosis if compared with an atherogenic diet and a control diet. Moreover 1% of CLA reduced an induced atherosclerosis in rabbits of 33% (Kritchevsky et al., 2000). Follow-up studies reported the efficacy of mixed CLA isomers, and a similar effect also exerted by the major CLA isomers *c9,t11* and t*10,c12* (Kritchevsky et al., 2004; Kritchevsky et al., 2002).
Studies on hamsters suggested that CLA or the individual isomers could be more effective against atherosclerosis when diet is high in saturated fat (Wilson et al., 2000; Mitchell et al., 2005; Valeille et al., 2005; Nicolosi et al., 1997).
However the effects of CLA isomers on atherogenic risk factors showed considerable variations, despite the evidence on atherosclerotic lesions (Lee et al., 1994; Nicolosi et al, 1997; Kritchevsky et al., 2000; Munday et al., 1999; de Deckere et al., 1999;

Valeille et al., 2004; Stangl et al., 2000[a,b]). Thus the mechanisms involved in the anti-atherosclerotic effects of CLA isomers have not yet been adequately addressed in both in vitro and in vivo studies.

Moreover atherosclerosis can be strictly related to obesity, metabolism disorders and hypertension.

Thus a positive effect of CLA on these factors could be suggested as an indirect CLA mechanisms affecting atherosclerosis.

CLA coul exert a role on the lipid metabolism through the action on PPARs, SREBPs and SCD. Peroxisome proliferator-activated receptors are ligand-activated nuclear receptors regulating the expression of genes that control lipid and glucose homeostasis, thus modulating the major metabolic disorders predisposing to atherosclerosis (Pineda Torra et al., 1999). Moreover, PPARs exert additional anti-inflammatory and lipid-modulating effects in the arterial wall, therefore being interesting molecular targets for the treatment of atherosclerosis (Marx et al., 2001).

Studies with pure isomers suggested that *c9,t11* is more effective than *t10,c12* in modulating key factors of lipid metabolism. Although both *c9,t11* and *t10,c12* isomers are ligands for PPARα, results showed that *c9,t11* isomer is the most effective activator (Moya et al., 1999).

c9,t11 isomer was also shown to down-regulate mRNA expression of SREBP-1c, whereas the *t10,c12* isomer did not showed any effect (Roche et al., 2002)

The complex relation between CLA and lipid metabolism will be discussed in the paragraph below.

Hypertension is another factor that could be associated with an increased risk of cardiovascular diseases.

t10,c12 CLA isomer or a mixture of the two isomers (50:50) were found to decrease the blood pression with a positive effect on hypertension by [a,b]Nagao et al. (2003) and Innoue et al. (2004).

Despite the CLA effect found on risk factors for cardiovascular health in animal studies, the results in the few human studies were discordant (Benito et al., 2001; Smedman et al., 2001; Petridou et al., 2003; Mougios et al., 2001; Noone et al., 2002; Tricon et al., 2004; Riserus 2002-2004; Moloney et al., 2004; Naumann et al., 2005; Gaullier et al., 2005).

Thus the beneficial effects of CLA on atherosclerosis need to be highlighted in more

clinical studies, and the understanding of the underlying mechanisms is required. Moreover the role of both the isomers should be identified.

7.3 Anti-adipogenic effects

As reported above, obesity is a pathologic condition related to risk factors such as cardiovascular disease, diabetes and cancer (Dilzer et al., 2012; Aminot-Gilchrist and Anderson, 2004). Therefore the CLA role in the control of body fat mass in animals and humans has been widely considered of interest (Park et al., 1997; Park, 2009). CLA was suggested to enhance the lipolysis or decrease the fatty acid uptake in adipocytes by Park et al (1997) and to increase the energy expenditure and fat oxidation (West et al., 1998, 2000; Terpstra et al., 2002; Ohnuki et al., 2001), decrease the adipocyte size (Tsuboyama-Kasaoka et al., 2000; Azain et al., 2000; Poulos et al., 2001) and the energy intake (West et al., 1998).

Moreover a basic role of CLA could be exerted in the inhibition of enzymes involved in fatty acid metabolism and lipogenesis (Park et al., 1999; Tasuboyama-Kasaoka et al., 2000; [a,b]Park and Pariza, 2001; [b]Park et al., 2000; Bretillon et al.,1999; Takahashi et al., 2002)

These hypotheses were supported by other studies that showed that CLA decreased fat mass and enhanced lean mass (Houseknecht et al., 1998; DeLany et al., 1999; West et al., 1998; Tsuboyama-Kasaoka et al., 2000;West et al., 2000; Ostrowska et al., 1999; Azain et al., 2000; Dugan et al., 1999; Atkinson et al., 1999; Halvorsen et al., 2000). *t10,c12* is the CLA isomer most involved in fat mass reduction, as reported from animal and in vitro studies (Park et al., 1999; Park and Pariza, 2007; Ryder et al., 2001; Choi et al., 2000; Navarro et al., 2003; [a,b]Riserus et al., 2002; Belury et al., 2003; Malpuech-Brugere et al., 2004; Herrmann et al., 2009). In vitro and in vivo studies showed the *t10,c12* effect in fat reduction through a decreased adipocyte size (Evans et al., 2000), an increased fat oxidation (Martin et al., 2000) and an inhibition of enzymes involved in lipogenesis (Choi et al., 2000; [a,b]Park and Pariza, 2001). Moreover [a]Nagao et al. (2003) recently showed that *t10,c12* isomer increased oxygen consumption and energy expenditure, more than the *c9,t11* isomer.

In the last study the effect of 6 weeks of supplementation of 0.5% LA, *c9,t11* CLA or *t10,c12* CLA in atherogenic diet-fed hamsters was investigated and a significant decrease of the fat mass in *t10,c12*-fed hamsters was obtained.

Also other studies reported similar results (Simon et al., 2006 and Obsen et al., 2012)

However it was also suggested that CLA was more effective in lowering fat mass when the diet was deficient in essential fatty acids (Kloss et al., 2005; Hargrave et al., 2004). As for the other CLA physiological effects, only few studies examined the effects of CLA or its isomers on body fat mass in humans. Moreover, as in the other cases, the results were not as dramatic as in animal trials and were discordant.

Zambell et al. (2000) and Petridou et al (2003) did not found any effect of CLA somministration (respectively 3g for 64 days and 2.1g for 45 days). However, two studies in healthy exercising humans (CLA, 1.8 g/day) and in overweight and obese subjects (from 1.7 to 6.8 g/day for 12 weeks) showed that CLA could decrease fat mass without significantly affecting body weight (Thom et al., 2001; Blankson et al., 2000). Thus exercise was suggested as an enhancer of the fat-lowering effects of CLA. Positive effects of CLA in human trials were also reported by Riserus et al. (2001) and Mougios et al. (2001). Moreover two long-term trials (12 month) conducted on healthy overweight humans by Gaullier et al. (2004, 2005) showed a significant decrease in body fat mass and the decrease in fat mass obtained in the second trial was maintained over a period of 24 month, thus suggesting that CLA might help in the maintenance of the reduction in fat mass. In addition Kamphuis et al. (2003) reported that CLA promoted lean body mass weight regain after a weight loss regiment and Watras et al. (2007) showed that a CLA supplementation for 6 months prevented the weight increase during the holiday season; also Whigham et al. (2007) and Park (2009) results suggested that a CLA long-term supplementation could affect the body fat mass. Anyway the human studies where CLA was supplemented through the intake of dairy foods did not showed the same healthy effects (Malpuech-Brugere et al., 2004; Desroches et al., 2005).

The review of Dilzer et al. (2012) underlined a possible effect of CLA on satiety in relation to the work of Gaullier et al. (2005).

As a matter of fact Kamphuis et al. (2003), Blankson et al. (2000) and Whigham et al. (2004) reported that CLA could affect hunger and satiety and reduce appetite, and Malpuech-Brugere et al. (2004) reported a reduction in average caloric intake by CLA supplemented subjects. However, the works of Lambert et al. (2007), Atkinson (1999); Gaullier et al. (2007); Iwata et al. (2007); Watras et al. (2007); Cornish et al. (2009); Norris et al. (2009) and Wanders et al., 2010 did not show any effect of CLA on these factors.

The mechanisms undergoing the CLA effect on body fat mass involved genetic

pathways. A study of Kang et al (2004) suggested that the CLA effects could be dependent by FAS and uncoupling protein (UCP) gene expression, and not by SCD, in according with Choi et al. (2004) and Ryder et al. (2001).

However a study of Obsen et al. (2012), reporting the predominance of *t10,c12* in lipid lowering, suggested that *t10,12* CLA could decrease SCD-1 activity, thereby reducing the MUFA needed for neutral and compound lipid synthesis.

As a matter of fact SCD knockout mice have lower synthesis of TG and cholesterol esters (Miyazaki et al., 2000), and SCD1-deficient animals also produce low levels of VLDL, suggesting that the rate of VLDL production might itself be influenced by SCD1 activity (Miyazaki et al., 2000, 2001).

Thus, inhibition of SCD1 activity could be one of the mechanisms involved in the lipid-lowering effect of CLA.

Also Choi et al. (2000) reported a repression of SCD gene expression in adipocytes by *t10,c12*. Moreover this effect was mainly exerted by *t10,c12* than *c9,t11* isomer (Choi et al., 2001).

Obsen et al. (2012) also found that *t10,c12* CLA could affect SREBP-1c pathway in bovine adipocyte, could decrease PPAR activity, thereby reducing the expression of adipogenic and lipogenic proteins needed for lipid biosynthesisand could finally increase inflammatory lipid metabolites or signals that antagonize glucose and FA uptake and subsequent metabolism (i.e., GLUT-4).

Other studies suggested instead that *c9,t11* isomer positively influences lipid metabolism by reduced synthesis and cleavage of hepatic SREBP-1.

The absence of dramatic evidence of CLA effect in human trials could be due to the CLA dosage that is lower in these studies than in animal studies. For example, in the study of Malpuech-Brugere et al. (2004) on mice, CLA was supplemented 0.5 w/w% (the same as 56 g CLA/day/70 kg), while in human studies the CLA supplementation ranged from 0.7 to 6.8 g/day as reported above.

Moreover, the human studies were mainly conducted with adults, while in animal trials the young subjects gave the most interesting results. Dilzer et al. (2012) also proposed the dietary regime as a factor affecting the CLA effects on fat mass: a test during dietary restriction did not show any reduction of body fat in mice ([a]Park et al., 2007) and humans (Whigham et al., 2004; Larsen et al., 2006; Diaz et al., 2008; Park, 2009), while CLA effects could be found during a weight gain period (Atkinson, 1999; Kamphuis et al., 2003a; 2003b; Whigham et al., 2004; Larsen et al., 2006; Watras et

al., 2007).
In addition, the same author reported other factors as subject weight status (normal, overweight, or obese), age, physical activity, physical condition, dietary interactions, or other medications or supplements that could be involved in the modulation of CLA effects in humans.

7.4 Effect against Insulin resistance

The animal and human studies regarding the CLA effects on glucose metabolism showed inconsistent results: most of them reported increased insulin resistance (IR) by CLA in normal animals but reduced insulin resistance in obese models (O'Hagan and Menzel, 2003; Wargent et al., 2005; Park and Pariza, 2009; Park, 2009), while effects of CLA on glucose metabolism in humans were inconsistent.
The works of Ryder et al (2001), Houseknecht et al. (1998), Nagao et al. (2003), reported positive effects of CLA on IR. Ryder et al., 2001 and Houseknecht et al., 1998 obtained a normalization of the glucose tolerance, an attenuation of the hyperinsulinemia and an increase in insulin sesitivity. Nagao et al. (2003) showed that CLA attenuated plasma glucose and insulin and prevented hyperinsulinemia by enhancing plasma adiponectin levels and mRNA expression.
Moreover comparisons among the *c9,t11* and *t10,c12* CLA isomers in the works of Ryder et al. (2001) and Houseknecht et al. (2003) suggested that *t10,c12* may be the isomer involved in beneficial effects at increasing IR levels.
Anyway Choi et al. (2004) reported that either the intake of *c9,t11* or *t10,c12* alone or a mixture o the two could enhance the glucose tolerance, while in some other studies the mixed isomer of the *t10*,*c12* and *c9*,*t11* CLA preparation was not associated with insulin resistance (Riserus et al., [a,b]2002, 2004; Moloney et al., 2004).
Moreover *t10,c12* CLA beneficial effects were also observed in a long term-treatment after an initial negative effect (Wargent et al., 2005).
In contrast the studies of Tsuboyama-Kasaoka et al. (2000), Roche et al (2002) and Ohashi et al. (2004) showed symptoms of lipoathrophic diabetes (mainly an induced IR) after CLA supplementation.
Regarding to human studies, Riserus et al (2002 a, 2004) found a decrease in insulin sensitivity after the supplementation of a CLA isomer mixture or individual isomers.
In contrast, CLA was found to improve insulin sensitivity in young sedentary humans

in the work of Eyjolfson et al. (2004) and an absence of negative effects of CLA on IR was found after long-term administration, unlike those of short-term administration (Whigham et al., 2004; Gaullier et al., 2004; 2005; 2007; Larsen et al., 2006; Syvertsen et al., 2007; Tarnopolsky et al., 2007; Watras et al., 2007; Racine et al., 2010; Sluijs et al., 2010), while only one study reported increased insulin levels (Gaullier et al., 2005). Moreover an improved glucose metabolism was reported also by Colakoglu et al. (2006), Gaullier et al. (2007) and Lambert et al. (2007).

The mechanism suggested to explain the CLA effect on IR are the increase of fatty acid β-oxidation, and/or the effects on adipokines and cytokines (Pariza et al., 2000; Sugano et al., 2001; Akahoshi et al.,2002; Yang and Cook, 2003; Bhattacharya et al., 2005; Chung et al., 2005; Park et al., 2007).

CLA seemed to be beneficial in rat models, while it showed negative effects in mice and human models, which could be associated with rapid loss of fat mass together with hepatomegaly. Thus more studies are needed to provide a conclusion on the CLA role on IR in animals and human, to clarify if *t10,c12* could be the only biologically active isomer and to define which mechanisms are involved.

7.5 Effect against Inflammatory response

Proinflammatory cytokines (TNF-α, IL-6, IL-1, etc.), anti-inflammatory cytokines (IL-10, IFN-γ), eicosanoids (prostaglandins, leukotrienes) are some of the key inflammatory mediators regulated by dietary intake of PUFA.

The effects of CLA on immune and inflammatory responses included the reduction of adverse effects of immune challenges, the reduction of colonic inflammation and of allergic type immune responses, and the modulation of the production of cytokines, prostaglandins, and leukotrienes (Cook et al., 1993; Miller et al., 1994; Belury and Kempa-Steczko, 1997; Whigham et al., 2001; Bassaganya-Riera et al., 2002; 2003; Yu et al., 2002; Luongo et al., 2003; Yang and Cook, 2003; Changhua et al.,2005; Bhattacharya et al., 2006; Hernandez-Diaz et al., 2010).

In vitro studies suggested a CLA effect on cell proliferation: CLA significantly inhibited cell proliferation and increased the expression of IL-2 and IFN-γ in the work of Luongo et al (2003), activated PPAR-γ in RAW 264.7 cells and decreased the cyclooxygenase (COX) 2 and TNF-α mRNA expression (Yu et al., 2002).

The effect of the single isomers has not been clarified yet: in the study of Yang and Cook (2003) mixed isomers of CLA and *c9,t11* isomer alone inhibited TNF-α production, while no effects were found by *t10,c12* isomer; *c9,t11* isomer was also found better than *t10,c12* in inhibiting eosinophil cationic protein formation (Jaudszus et al., 2005), while Changua et al. (2005) concluded that inhibitory activity of CLA on pro-inflammatory cytokines was related to *t10,c12* isomer. However [b]Yamasaki et al. (2003) reported that *c9,t11* isomer significantly increased TNF-a production compared to control and *t10,c12*-fed mice.

181 study indicated that there was no difference in activity between *c9,t11* and *t10,c12* isomers as far as effect on immune function is concerned.

Studies in cancer cells showed no effect or a decrease in prostaglandin and other inflammatory mediators production (Park H.S. et al., 2004; Miller et al., 2001; [a]Park Y. et al., 2000; Ma et al., 2002; [b]Kim et al., 2002).

Animal studies reported interesting results. CLA was found effective in lowering TNF-α and IL-6 in serum of mice (Bhattacharya et al., 2005) and Akahoshi et al. (2002) reported that a dietary intake of 1% CLA decreased serum TNF-α and leptin levels comparing to LA. In the work of [a]Yamasaki et al. (2003) 1.5% CLA decreased serum TNF-α irrespective of fat content of the diet.

Moreover pigs fed with 2% CLA, showed a reduction in growth depression, the prevention of production and mRNA expression of IL-6 and TNF-α, and an increase in PPAR-γ and IL-10 expression (Changua et al., 2005).

Two studies by Yang et al. (2000, [a]2003) confirmed that CLA has some protective effect against down-regulating autoimmunity. In contrast, a study in rats fed with different diets and 1% CLA for 3 – 4 weeks reported no effects on serum levels of leptin and TNF-α (Sugano et al., 2001) and in the work of Tsuboyama-Kasaoka et al. (2000) TNF-α mRNA expression was increased by 12-fold in adipocytes isolated from mice fed with 1% CLA. Moreover Poirier et al. (2006) reported that the *t*10,*c12* isomer induced inflammatory responses in mice white adipose tissue.

Only few studies were conducted regarding the effects of CLA on immune function in humans. Moreover the effects of CLA on immune and inflammatory responses in humans were not consistent.

The study of Kelley et al. showed no effect of 3.9 g/day CLA supplementation for 9 weeks on indices of immune status of women (Kelley et al., 2000,2001).

A subsequent study in men showed similar findings when *c9t,11* and *t10,c12* CLA isomers, respectively in proportions 50:50 or 80:20, failed to alter immune response like TNF-α, IL-6, IFN-γ, IL-2, IL-4, PGE_2 and lymphocyte proliferation (Albers et al., 2003).

Moreover Nugent et al. (2005) showed that supplementation with similar ratio of CLA isomers did not show any immunological benefits compared to control LA.

In contrast Song et al. (2005), investigating the effects of 3 g/day supplementation of CLA (50:50 *c9,t11* and *t10,c12*), found plasma levels of IgA, IgM and anti-inflammatory cytokine IL-10 to be increased with concomitant decrease in levels of IgE, and proin- flammatory cytokines, TNF-α and IL-1h.

Beneficial effects by CLA supplementation were also found by Albers et al. (2003) and Turpeinen et al. (2008). In the first work a initiation of the response to hepatitis B vaccination was improved by CLA supplementation (*c9,t11* and *t10,c12* 50:50).

Turpeinen et al. (2008) reported that the supplementation of 2 g/d *c9*, *t11* CLA for 12 weeks showed improved feeling of well being and less sneezing in subjects affected by pollen allergy (Turpeinen et al., 2008).

In vitro and in vivo studies indicated the CLA mediatory effects on cytokine and prostaglandin production. It has still to be established if *t10,c12* isomer could be more anti-inflammatory compared to *c9,t11* isomer, as reported in some studies.

Moreover clinical studies reported contrasting results. Thus further studies on humans are required before CLA isomers can be recommended to improve immune function.

7.7 Bone health

A CLA improvement on bone mass in animal models was reported, even if the effects were not consistent (Park et al., 1997; Watkins et al., 1997; Li and Watkins, 1998; Turek et al., 1998; Li et al., 1999; Park et al., 1999; Thiel-Cooper et al., 2001; Demaree et al., 2002; Kelly et al., 2003; Ostrowska et al., 2003; Berge et al., 2004; Kelly and Cashman, 2004; Weiler et al., 2004; Banu et al., 2006; Burr et al., 2006). This inconsistency was suggested to be due in part to the interaction between CLA and dietary calcium (Park et al., 2008).

In 1999 Li et al. reported that CLA regulated bone metabolism by modulating IGF-I and IGFBP in young male SD rats after 42 days of treatment. Then in 2003, Kelly et al.

showed that the supplementation of CLA could enhance calcium absorption.
In another study CLA supplementation for 8 weeks showed reduced bone resorption rates (Kelly et al., 2004). An increased bone mass was also found by Banu et al. (reported by Bhattacharya et al, 2006).
Clinical studies of Kreider et al. (2002) and Brownbill et al. (2005) reported beneficial effects of CLA on bone mass; anyway no significant changes in the markers of bone turnover, bone mass and strength were found (Kreider et al., 2002; Doyle et al., 2005) in clinical studies.
Moreover Racine et al. (2010) reported potential adverse effects of CLA on bone mineral content.
The main mechanism involved in CLA effect on bone health might be the enhancement of calcium absorption, as suggested from animal and human CaCo2 cell studies (Roche et al., 2001; Jewell and Cashman, 2003; Kelly et al., 2003; Jewell et al., 2005; Murphy et al., 2006; Park et al., 2006).
Thus animal models clearly showed that CLA could increase bone mass in rats and mice. However no consistent results were found in the few clinical studies performed and further studies regarding CLA and calcium absorption are needed to determine the effects of CLA on bone health.

VIII. CLA ANALISYS

Lipid estraction

The lipid extraction was performed according to the modified Folch's technique (Christie, 1989). 30 g samples were mixed with 300 ml chloroform–methanol mixture (2:1, v/v); after homogenising in a Ultra-Turrax T25 homogeniser (Janke & Kunkel, GmbH & Co, Staufen, Germany), the mixture was agitated for 60 min and was then filtered into a separator funnel through filter paper (Albet folded circles, 130 cm, extra rapid). Seventy-five ml of saturated NaCl solution were added to the filtrate; chloroform phase was subsequently recovered, dehydrated with anhydrous sodium sulfate (Na_2SO_4) and dried with a rotary evaporator at 40 °C under vacuum.
Two quantities of one hundred milligrams each (fat samples A and B) were taken from the fat extracted for every sample and transferred in two different glass-stoppered test-tube of approximately 10 ml capacity for the preparation of the fatty acid methyl esters.

Preparation of c9,t11 and t10,c12 methyl esters

The esterification was in accordance with the method described by Bannon, Craske, & Hilliker (1985) with some differences. The fat samples A were dissolved in 2 ml of hexane solution containing an internal standard (IS, nonadecanoate methyl ester acid, 0.3 mg m-l), whereas the fat samples B were dissolved in 2 ml of hexane solution without addition of IS. All the fat samples A and B were then esterified with 2N methanolic-potassium-hydroxide (100 µl). The mixtures were shaken vigorously for 30 s and allowed to react for a total of 6 min at room temperature (ca. 28°C). The catalyst (KOH) was neutralized immediately by adding 2N hydrochloric acid (100µl) with shaking, to the methyl orange end-point. The hexane phase, containing the *c9,t11* and *t10,c12* methyl esters, was separated by centrifugation and subjected to gas chromatography.

Gas-chromatographic analysis

c9,t11 and *t10,c12* methyl esters were quantified using a GC (Varian 430) equipped with a flame ionization detector and a CP-Select CB capillary column for fatty acid methyl esters (FAME) (100 m* 0.25 mm i.d.; 0.25 mm i.d; 0.25 µm film thickness;

Chrompack, Varian, Inc., CA). GC oven parameters and gas variables were: isotherm analysis at 175°C for 65min; temperature of injection 250°C; detector temperature 250°C; injection volume 1µl; gas carrier He and its flow rate 1.5 ml min-1. *c9,t11* and *t10,c12* isomer peaks were identified by comparison with the retention times of reference standards (methyl *c9,t11* and *t10,c12* octadecadienoate). C19:0 (IS) was eluted at 25.86 min, *c9,t11* at 33.72 min and *t10,c12* at 34.77 min.
Since a peak overlapping with the C 19:0 (IS) was verified by previous analytical quality control, two GC analyses were conducted (esterified fat samples A with IS and B without IS) for each sample. This procedure permitted us to measure the area of the interfering peak which was then subtracted from the C 19:0 area to calculate the *c9,t11* and *t10,c12* contents in the samples.
The column resolves five distinguishing peaks in the CLA region on the chromatogram of fat: *c9,t11+t7,c9+t8,c10*; *t11,c13+c9,c11*; *t10,c12*; *t11,t13*; *t9,c11* CLA isomers (Blasko et al., 2009; Kraft, Collomb, Mockel, Sieber & Jahreis, 2003; Kramer, Hernandez, Cruz-Hernandez, Kraft, Dugan, 2008). The important *cis,trans* isomers of CLA usually elute in a region of the chromatogram that is free from other fatty acids (Blasko et al., 2009). In our study, the co-elution of the *c9,t11+t7,c9+t8,c10* triplet was not checked owing to commercial unavailability of the *t7,c9* and *t8,c10* isomer standards. Nevertheless, *t7,c9* and *t8,c10* isomers occurrence in very low amounts in meat and dairy products. In fact, the *t7,c9*, which is the second most abundant CLA isomer in ruminant fat, normally can amounts up to 7% of total CLA (Kraft et al., 2003). Thus the hypothetical *c9,t11+t7,c9+t8,c10* peak was considered only as *c9,t11* peak.

The *c9,t11* and *t10,c12* content, expressed in mg g-1 fat, was determined by the following formula:

*CLA isomer (mg g-1 fat)=(area CLA*conc IS*CFCLA)/(areaIS*conc.fat*1.04)*

Where "CLA" means *c9,t11* or *t10,c12* isomer; ''1.04'' is the conversion factor from methyl ester to fatty acid and ''CF'' the isomer correction factor obtained from an average of 10 injections of a mixture containing 0.025g standard isomer (*c9,t11* or *t10,c12*) adequately methylated and 0.010 g nonadecanoate methyl ester; and "area IS"

is the peak area of the C 19:0 in the fat sample A subtracted the peak area eluting at the retention time of the C 19:0 in the fat sample B. The CF correction factor was determined as follows:

$CF_{CLA}=(\text{area}_{STD}/\text{area}_{CLA})*(\text{conc}_{CLA}/\text{conc}_{STD})$

MANUSCRIPTS

IX. MANUSCRIPT 1

Manuscript in press in "La qualità degli alimenti di origine animale e la salute umana".
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Dairy food and health

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Abbreviazioni: CLA, conjugated linoleic acid; CLNA, conjugated linolenic acid; IGF-I and II, insulin-like growth factor I and II; PPAR, peroxisomal proliferator activated receptor; DMBA, dimethylbenzantracene; NMU, *N*-nitroso-N-methylurea; BAT, brown adipose tissue; WAT, white adipose tissue; SRBP, sterol regulatory binding protein; VEGF, vascular endothelial growth factor.

1. Introduction

The definition "Dairy food" is referred to milk and all its products, like butter, cheese, yoghurt and fermented products, creams.

The dairy foods are some of the main components of the Mediterranean diet, that has been recognised "Unesco Asset" since the start of this year. Dairy foods are sources of high quality proteins and of different kinds of fats.

A lot of studies have been carried out in the last decade about the effects of dairy foods constituents on human health: this article want to unify the discoveries to collect some global conclusions about the intake of dairy foods and human health.

2. Health-related components of dairy foods

There are some dairy food components having specific roles in human health, and the most important are the Conjugated Linoleic Acids, the saturated fatty acids, the Insulin-like Growth Factor I (IGF) and the estrogens, the calcium and vitamin D.

Conjugated Linoleic Acids (CLA) is the common name given to the family isomers of Linoleic Acid, a group of double-unsaturated C18 fatty acids. Among them, the most important isomers are the C18:2 *c9,t11* CLA and the C18:2 *t10,c12* CLA. These fatty acids belong to the ω6 group, and togheter with ω3 are a basic requirement for health: the first group is needed for cell walls, while the second exerts a positive effect on cardiovascular health and on cell structure, because they can act against atherosclerotic plaques.

Beside unsaturated fatty acids, some dairy products, like whole milk and many types of cheeses, contain also saturated fatty acids that can have a negative role on health.

Saturated fatty acid were recognised as one of the most important causes of cardiovascular diseases, dyslipidemy, and some neoplasias (Moorman et al., 2004).

The fatty acid profile of milk could be altered by the diet of the animal, reducing saturated fatty acids and increasing α-linoleic acid to exert a positive role on cardiovascular health.

The IGF I is a peptide also known as somatomedin C, that is usually present in in normal, untreated human and bovine milk. It is a potent mitogen and has a proliferative role in breast cancer cells in vitro (Outwater et al., 1997).

The IGF I action is often related with the presence of estrogens: they can increase IGF-I in breast cancer cells and IGF-I can work in synergy with them (Outwater et al., 1997).

Estrogens are present in milk, both in free form and in protein-bound form. Free estrogens have been found in commercial, pasteurized bovine milk and skim milk.

3. Physiological role of CLA

CLA and body weight

It was showed that CLA could reduce the body mass fat and increase the lean body mass.

Some mechanisms involved in fat reduction were suggested: increase in energy expenditure and decrease in energy intake; increase of the fat oxidation; decrease of the adipocyte size and inhibition of some enzymes involved in fatty acid metabolism and lipogenesis (Bhattacharya et al., 2006).

Some studies with purified isomers made clear that the C18:2 *t10,c12* CLA isomer is related with the last three functions, while the C18:2 cis-9,trans-11 CLA is not involved and is also less powerful in increasing oxygen consumption and in energy expenditure.

Uncoupling proteins (UCP) are the key regulators of the energy expenditure and it was suggested that UCP-2 up-regulation expression was mediated by CLA in white adipose tissue. Another potential mechanism explaining the increase of energy expenditure by CLA is the increase in catecholamines (Bhattacharya et al., 2006).

All these studies were performed on animal models, while clinical studies showed positive effects of CLA supplementation in reducing body fat mass, but the magnitude of this improvement was lower than ones observed in animals (Bhattacharya et al., 2006).

CLA and atherosclerosis

Another healthy function of CLA can be related to the cardiovascular system: CLA was suggest to be a protective molecule against atherosclerosis.

Accumulation of lipids can induce chronic inflammation by promoting macrophage infiltration and activation (Stachowska et al., 2010).

During inflammations disorders, the macrophages accumulate within the arterial neointima: so this fact becomes the major contributor of the atherosclerotic plaque.

1st stage: appearence of dysfunctional endotelial cells, whose activated adesion molecules and expressed chemokines recruit circulating monocytes and lymphocites into the intima;

2nd stage: accumulation of LDL in the arteria wall, where it undergoes modification by macrophages ⟶ these modifications increase LDL uptake by macrophages through the overexpression of CD36 and SRA

CD36 and SRA are the most important scavenger receptors on the macrophages surface: during inflammation, circulating LDL are taken up by macrophages through these receptors (Stachowska et al., 2010).

The atherogenic plaque can be solved by the activation of PPAR (peroxisome proliferator activated receptors) and in particular the activation of PPARγ, that increase adiponectin synthesis and thus down regulate the pro-inflammatory genes (Zhang et al., 2011; Kadoglou et al., 2008; Delerive et al., 1999).

The mechanisms suggested were based on the role of CLA on peroxisome proliferator-activated receptors (PPARs), on stearoyl-COA desaturase (SCD) and on sterol regulatory element binding proteins (SREBPs).

PPARs are ligand-activated nuclear receptors regulating the expression of genes that control lipid and glucose homeostasis.

There are two main PPARs involved in this mechanism: PPAR α, that has a key role in expression regulation of genes involved in fatty acid oxidation and energy homeostasis, and PPARγ that induces the expression of genes that promote lipid storage and controls the CD36 expression, that allow the endocytose-mediated uptake of oxidated LDL by macrophages.

The down-regulation of PPARγ exerted by t10,c12 isomer reduced the CD36 macrophage receptor expression and the fat deposition in macrophages, thus reducing the *foam cells* formation (Stachowska et al., 2010). The reduction of atherosclerotic processes by *c9,t11* CLA is instead related to a down-regulation of pro-inflammatory genes (Ringseis e Eder, 2009).

C9,t11 is more effective in modulating PPARs, while both c9,t11 and t10,c12 are legands for the PPAR α.

SREBPs regulate fatty acid and triglicerids synthesis. About this hypothesis was found that a reduction of the synthesis and cleavage of hepatic SREBP-1 made by c9t11 isomer positively influences lipid metabolism (Bhattacharya et al., 2006).

Another hypothesis is related to SCD: it suggest that the SCD activity can be inhibited in some cases by both the c9,t11 and t10,c12 CLA isomers, making a lipid lowering effect (Bhattacharya et al., 2006).

These results were obtained from animal and *in vitro* studies, while clinical studies gave disappointing results.

Initial studies on rabbits showed that after feeding with 14% fat (high fat) and 0,1% cholesterol, the CLA-fed rabbits had lower atherosclerosis in aortas. It was then suggested with other studies that only the t10,c12 isomer could be active against atherosclerosis.

CLA and cancer

Gastrointestinal cancer

The studies made about CLA and inhibition of gastrointestinal cancer are mainly *in vitro* and animal studies.

The clinical studies reported about the inhibition of HT-29 colon cancer cells because CLA induces apoptosis. In these studies the t10,c12 CLA was shown as the only agent acting against the cancer, inhibiting IGF-II.

It was also found that CLA inhibits metastasis of gastric and colon cancer cells inoculated in mice; 1% CLA can decrease colon cancer in rats decreasing prostaglandins PGE2 levels.

A Scandinavian study (Larsson et al., 2005) observed that an increase of two high fat-dairy food portions reduced the colon cancer incidence from 4 to 13%. The authors only slightly attribuited this protective effect to CLA.

Anyway, the inverse relationship between dairy product consumption and intestinal cancer is also reported by other epidemiological studies and a lot of factors are involved (pH, probiotic cultures, immuno-modulator peptides, Ca) (Elwood et al., 2004).

Breast cancer

CLA is found to have a dual ability: act as a preventive and therapeutic agent in a number of rodent and human tumor model systems.

CLA could inhibit mammary carcinogenesis by acting on normal or initiated epithelial cells within ducts, alveoli, terminal end buds, or on transformed epithelial cells to inhibit their growth, alter their differentiation, and/or to introduce cell These effects could be direct, via CLA delivery through blood flow, or indirect, through CLA release from the mammary adipocytes, and/or CLA alteration of the mammary stroma (Ip M. et al., 2003; Banni et al., 2001).

In particular, CLA has been shown to inhibit rat mammary carcinogenesis induced by dimethylbenzanthracene (DMBA) and N-nitroso-N-methylurea (NMU): CLA was

found to be effective when given concurrently with the carcinogen, suggesting that one activity of CLA may be the inhibition of carcinogen activation (Ip M. et al., 2003)
Dietary CLA decreases the number of epithelial target cells in the mammary gland and stimulates apoptosis of preneoplastic lesions (Banni et al., 2001).
Some studies indicated that a dietary level of 0.1% CLA is sufficient to produce a significant inhibition of carcinogen induced rat mammary tumors (Ip C. et al., 1991)
In one of the studies about the rat mammary carcinogenesis model, a mixed isomer preparation of CLA (approx. 1:1 c9,t11 and t10,c12, with minor amounts of other isomers) was used. The effect was dose-related: low effect at 0.05% (w/w) and maximum effect at a dietary level of 1% (independent of the type or level of fat in the diet). CLA was equally effective when provided in the form of triglyceride or free fatty acid.
c9,t11 and t10,c12 isomers of CLA were found to be equally effective in inhibiting the development of NMU-induced preneoplastic lesions and tumors in the mammary gland, and the equal efficacy of the two isomers was then confirmed in a mammary metastasis study (Ip M. et al., 2001).
The results of some diets are described in table 1.
It was tried to compare different experiments in an observational way.

Table 1. CLA dose effect (g/100 g diet) on breast cancer incidence in rats. Values within the same row were provided in different studies using the same CLA concentration.

Cla concentration in diet (%)	**CORRECTED INCIDENCE %**				
0	100				
0,05	103,57[a]				
0,1	75[a]	128,57[b]	91,43[c]		
0,25	60,71[a]	114,29[b]	85,71[c]		
0,5	75,03[a]	64,29[a]	65[a]	53,57[b]	57,14[c]
0,8	53,57[d]	57,14[e]	60,71[f]		
1	53,59[a]	43,75[a]			
1,5	50,05[a]	37,5[a]			

[a] *c9,t11 (42%)+ t10,c12 (46%) (Hubbard et al., 2003)*

[b] *c9,t11 only*

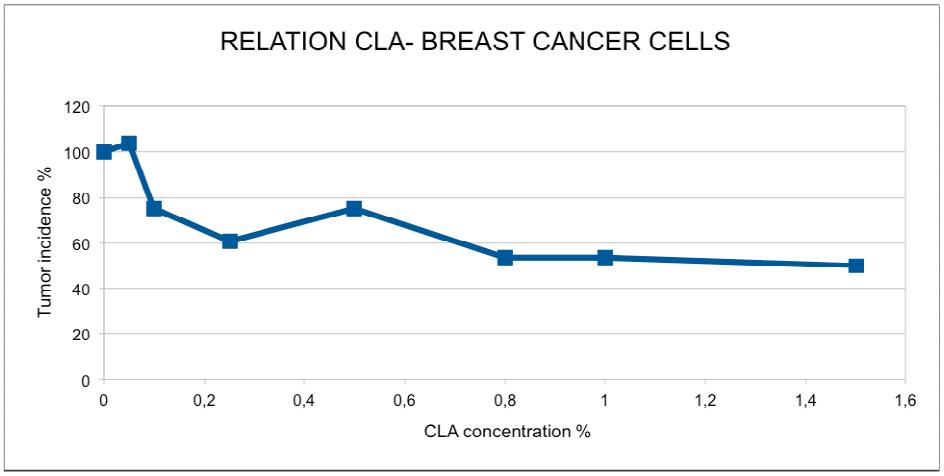
[c] *t10,c12 only*

[d] *c9,t11 92% (Jiang et al., 2009)*

[e] *c9,t11 + c9,c11 (Jiang et al., 2009)*

[f] *c11,t13 + c9,t11 + t10,c12 + t8,c10 (Jiang et al., 2009)*

The relation between CLA intake and breast cancer incidence is showed in the figure below. Cancer incidence is inversely related to CLA intake as long as 0.25% level of CLA supplementation. Higher levels than 0.25% did not improved the anti-tumoral effect of CLA.



A recent study showed that a mixed CLA preparation markedly change the composition of the mouse mammary fat pad, an effect that is completely due to the t10,c12 isomer (Masso-Welch et al., unpublished data cited in Ip M. et al., 2003). In this study a CD2F1 mouse was fed t10,c12 CLA at levels of 0.5 and 1.0% in the diet and the results were the complete abrogation of the brown adipose tissue (BAT) component of the mammary fat pad, and the significantly reduction of the white adipose tissue (WAT) compartment.

This is the result of the induction of apoptosis, that can be a consequence of the marked induction of apoptosis of capillaries into the BAT and WAT.

No increase in apoptosis of mammary adipocytes, or the stromal capillaries, was instead noted in mice fed the c9,t11 CLA isomer. Indeed, when fed at the 1% level, the c9,t11 CLA isomer increased the proportion of BAT in the mammary gland, maybe due to the CLA stimulatory effect on the adipogenic differentiation of the multipotent stromal-vasculare cells in the mammary gland (Ip M. et al., 2003).

It was found that the two main CLA isomers have different mechanism to inhibit the cell proliferations (Chujo et al., 2003; Hubbard et al., 2003)

The mechanism by wich CLA inhibits mammary carcinogenesis includes the action on the vascular endothelial growth factor VEGF-A.

VEGF is a cytokine that is known to stimulate vascular permeability and migration, proliferation and apopthosis of endothelial cells: it can increase the invasiveness and the growth of breast cancer cells.

A mix of the CLA isomers can decrease the VEGF serum levels and can act against one of the VEGF receptors, affecting the mammary carcinogenesis directly on epithelium and indirectly acting against the angiogenesis (Masso-Welch et al., 2002; Ip M. et al., 2003).

In vitro studies showed that CLA is cytotoxic and induce lipid peroxidation in MCF-7 cell line (human breast cancer cells).

CLA at a concentration 3.5×10^{-5} M selectively inhibits proliferation of ER (estrogen) positive MCF-7 cells as compared with ER negative MDA-MB-231 cells. Cell cycle studies indicated that a higher percentage of CLA treated MCF-7 cells remained in the G0/G1 phase as compared to control and those treated with linoleic acid (LA). Many anti-estrogens are known to possess this property and are able to block the cell cycle at the G0/G1 phase.

Results indicated that MCF-7 cells grown with CLA for 4 days began to proliferate upon their return to normal media: the growth inibitory action of CLA on these cells is reversible.

Isomer t10,c12 CLA inhibits cell proliferation if induced by insulin and estrogen, while c9,t11 CLA has a different mechanisms to inhibit cell proliferation. In the absence of CLA, or upon its withdrawal from media, the normal mitogenic pathway continues to operate resulting in increased proliferation of cells.

In a recent study has been shown that CLA isomers down regulate estrogen receptor alfa expression both at mRNA level and at protein level, and reduce the link between a nuclear protein and a estrogen response element. Thus, CLA isomers have significant antiestrogenic properties (Bhattacharya et al., 2006).

Focusing on these experimental results it can be hypothesized that CLA inhibits MCF-7 cell growth by interfering with the hormone regulated mitogenic pathway (Durgam et al., 1997).

Clinical studies gave contrasting results.

In some cases it was reported that an increased CLA intake via whole milk decreased the risk of breast cancer in women, and it was further shown that dietary CLA is also effective in the prevention of DMBA induced mammary tumors (Aro et al., 2000; Knekt et al., 1996); in other cases CLA content in diet was not associated with a lower risk of breast cancer (Voorrips et al., 2002).

Prostate cancer

It was shown that CLA has a antiproliferative effect *in vitro* and *in vivo*. Some in vitro studies showed that the t10,c12CLA is more useful than c9,t11 against prostate cancer. The in vivo experiments are few, but a decrease in tumoral metastasis in animals locally injected with cancer cells and fed CLA was observed, while no difference could be detected in control animals.

It was suggested that the mechanism by which CLA can inhibit the prostate cancer consisted mainly in modulating the apoptosis and the cell cycle as it was refrerred also about breast cancer and gastrointestinal cancer.

The actions of CLA isomers are direceted on different molecules: t10,c12 CLA can modulate the genes involved in apoptosis and in cell cycle control, while c9,t11 CLA can regulates some genes involved in arachidonic acid metabolism, thus attenuates the eicosanoid synthesis.

CLA isomers can infact reduce the COX and LOX gene expression (c9,t11), related to the arachidonic acid methabolism; they can decrease bcl-2 expression and induce apoptosis in PC3 cells, thus regulating cell cycles and apoptosis (Ochoa et al., 2004).

Angiogenesis

One important action of both CLA isomers against cancerogenesis is based on the control of angiogenesis.

Some *in vivo* studies reported that both the isomers can decrease VEGF serum levels and Flk-1 protein in the mammary gland, due to control angiogenesis.

It was also shown that both 1%CLA and 2% CLA diets significantly decrease angiogenesis, without a significant difference from each other, not only acting on VEGF, but also inhibiting the formation of functional blood vessels (Masso-Welch et al., 2002).

To understand the CLA inhibition of angiogenesis some mechanisms were hypothesized:

1) decrease of the initial negative cellular alterations and angiogenic functional blood-vessels; 2) reduction of the cellular network; 3) inhibition of microcapillary networks *in vitro;* 4) regulation of systemic and local VEGF and its receptors.
Some studies also underlined the presence of another potential mediator of CLA effects against angiogenesis. It was infact shown that leptin, a proangiogenic hormone, decreased in plasma levels with the administration of t10,c12 CLA.

CLA and insulin resistance (IR)
CLA attenuated plasma glucose and insulin and prevented hyperinsulinemia by enhancing plasma adiponectin levels and mRNA expression in white adipose tissue from ZDF rats.
Only long term treatment could improve insulin sensitivity and glucose tolerance, while at first the CLA treatment may have a bad effect on IR (Bhattacharya et al., 2006).
Some clinical studies showed that the t10,c12 CLA induces hyperinsulinemia in obese individuals, while in other studies CLA was found improving insulin sensivity (Bhattacharya et al., 2006).

CLA and anti-inflammatory response
Either CLA and CLNA have a benefical effect on inflammations:
- down regulation of eicosanoid production
- increase of PPAR mediated anti-inflammatory response
- suppression of inflammatory response through the regulation of cell transcription factor nuclear factor k B (NF-kB)
- reduction of expression of pro-inflammatory proteins (TNF-α; Leukin)
In animals CLA induce negative regulation of the expression of proinflammatory genes and activation of apoptosis in the atherosclerotic lesion [4].

CLA intake
According to Kelley et al. (2007) the intake of CLA in humans, corresponding to the effective levels in animals range from 5 to 50 g/day. A recent paper from Mushtaq et al. (2010) reported a daily intake of 97,5 mg in 18 british volunteers, while Aro et al. (2000) estimated a CLA intake in finnish women from 126.8 to 142.3 mg/day. Data on

CLA intake by italian population are not available, however on the basis of these two works (Aro et al., 2000; Mushtaq et al., 2004), we need to increase CLA content in milk and dairy foods as a tool to make available foods that fit well with the need of a more and more health-conscious consumer.

4. Phisiological role of saturated fatty acids

A large intake of dairy food can mean a high introduction of fat, expecially in terms of saturated fatty acids (Moorman et al., 2004) and cholesterol.

The negative effects of this type of fat can be mainly two: the increase in LDL and in cardiovascular problems, and the increase in circulating estrogen concentration.

In the Seven Countries Study was reported that a high saturated fat intake was in strong relationship with coronary death rates.

In other studies was also discovered that different saturated fatty acid had different effects on LDL levels: while stearic acid (18:0) does not affect cholesterol levels if compared with monounsaturated oleic acid (18:1), the saturated fatty acid shorter than C18, like C14 and C16 tend to increase plasma levels of cholesterol and LDL.

It was thus shown that replacing the saturated fats with poli-unsaturated ones was clearly effective in lowering serum cholesterol, and so in reducing the risk of coronary heart diseases (Hu et al., 2001).

5. Phisiological role of insulin-like growth factor and estrogens

Estrogen and IGF-I are present in human and bovine milk, in free and bound form, they cannot be destroyed with pasteurization and they can only be reduced with milk filtration.

Insulin like growth factor IGF-I and IGF-II, also known as somatomedin C and A, are peptides acting as growth factor in local tissues.

They are suggested to be potent mitogens, normally present in human and bovine milk.

It was reported that the bovine GH administration to dairy cows increases the concentration of IGF-I in milk (Outwater et al., 1997).

The IGF-I is supposed to be a contributor to breast cancer cells proliferation, because of its action on the IGF-I receptors and binding proteins. Some *in vitro* studies revealed that breast cancer cells respond to nanomolar concentrations of IGF-I and it was shown

that these neoplastic cells have more receptors for IGF-I than the normal mammary tissue.

IGF-I also causes changes in cell cycle and oncogenes like *c-fos*.

The direct consequence of these IGF-I actions may be the non-controlled growth of the cancer.

Some other studies reported again that IGF-I is involved in cell transformation because removing or blocking its receptors can eliminate viral or cellular oncogene-induced malignant transformation (Moorman et al., 2004).

It has to be considered that IGF-I can work in synergy with other growth factors, making the transformed cells more responsive to their signals.

The estrogens have the best synergy with IGF-I: they were found to increase the IGF-I level in human breast tissue and IGF-I was called estromedin itself, because it mediates the estrogen effects.

In some studies an high plasma level of estrogen was linked with breast cancer incidence (Outwater et al., 1997; Moorman et al., 2004).

Thus IGF-I can stimulate the growth of human breast cancer cells, acting in sinergy with estrogens.

Another important consideration leads to the absorption of IGF.

Intact IGF-I can be absorbed by the gastro-intestinal tract and can travel through the bloodstream, exerting its mitogenic effects on local tissues, so the concerns about this peptides are not only related to the IGF-I already present in our body or produced thanking to the estrogen, but also to the growth factors introduced by whole milk intake.

6. Phisiological role of Calcium and Vitamin D

Calcium and vitamin D are strictly related each other: vitamin D regulates Ca absorption and metabolism. Much of the evidence showing the protective role of Ca against cancer are related to linkages with the vitamin D.

It was shown that the active form of vitamin D markedly affects cell growth processes and development; then it was also reported about a specific role of this molecule in the differentiation of the mammary gland (Moorman et al., 2004).

Vitamin D can in fact inhibit the proliferation of cell cycle, arresting it at phase G0/G1, like CLA. In this way it can down-regulate several promoting factors as IGF and up-regulate some negative growth factors.

Then the vitamin D can also exert a regulation of the cell cycle, inducing apoptosis, cell shrinkage, chromatin condensation and DNA fragmentation (Moorman et al., 2004).

The alone Ca effects are not so evident: in some studies was shown that the inverse relation between calcium and cancer was statistically significant only at the highest vitamin D intake (Cho et al., 2004) but it was shown that also Ca in itself has an apoptotic effect, thus protecting against the cancer (Alvarez-Leon et al., 2006; Moorman et al., 2004).

7. Conclusion

A lot of studies were carried out to find whether an inverse relation between dairy foods and cancer could be demonstrated.

Many studies showed the inverse relation between dairy foods intake and cancer (Cho et al., 2004; Knekt et al., 1996).

We have to discriminate between different types of cancer. Milk was revealed as a protective factor against colorectal cancer in some articles (Knekt et al., 1996; Alvarez-Leon et al., 2006; Moorman et al., 2004; Cho et al., 2004).

In one of these experiments was discovered that an increase of 500g/day in milk consumption was associated with a 12% reduced risk of colorectal cancer.

Some studies showed the protective effects of dairy foods against breast cancer (Ip C. et a., 1996; Knekt et al., 1996;Shin et al., 2002; Cho et al., 2004) expecially about fermented products: it was underlined the absence of any positive relation between fermented milk and dairy products and breast cancer risk (Van't Veer et al., 1989).

The observational analysis we have done showed that, comparing some animal studies, a trend can be found between breast cancer risk and intake of CLA.

Unfortunately not enough clinical studies are available to prove the same trend on humans.

In few studies the protective effects of dairy foods against this kind of cancer cannot be demonstrated, and was instead found a positive relation between the two (Voorrips et al., 2002; Alvarez-Leon et al., 2006).

In dairy foods the concurrent presence of some health protective factors and negative factors is clear.

Thus the epidemiologic studies nowadays are not able to establish which of these factors is the more relevant for human health.
It can be shown that some studies revealed positive effects of dairy foods that cannot be statistically demonstrated and some results cannot be clearly connected with a specific component of these foods.
Concluding we can say that dairy foods are essential for body metabolism and some components are probably involved in healthy processes.
The major effects against cancer are related to the synergy between CLA, vitamin D and calcium.
These molecules can also be involved in other healthy effects, like prevention against atherosclerosis, fat body mass reduction and insulin resistance control.
The CLA isomers have different targets and mechanisms of action, but they can surely regulate cells proliferation and estrogens or growth factors effects.
The overview on these results thus suggests that dairy foods (with a correct intake) are important to protect us against some of the main concerns of our times, but other studies are needed to say that they are surely safe, even their high saturated fat content.

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X. MANUSCRIPT 2

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Conjugated linoleic acid isomer (*cis9,trans11* and *trans10,cis12*) content in cheeses from Italian large-scale retail trade

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Abstract

The aim of the work was to complete data obtained in previous studies with a survey on *cis9,trans11 (c9,t11)* and *trans10,cis12 (t10,c12)* conjugated linoleic acid (CLA) content in cheeses collected from Italian large-scale retail trade. This is an integrative part of a whole study characterizing food CLA content, with objective of estimating daily CLA intake of Italian consumers. Among the sampled cheeses (n=102), Gruyere and Feta (10.21 and 8.50 mg g^{-1}fat, respectively) had the highest ($P<0.05$) *c9,t11* contents. Furthermore, cheeses with long-ripening period (>180 d) showed higher *c9,t11* values than those with a shorter maturation period. The *t10,c12* CLA isomer was almost absent, being detected only in Gruyere, Stracchino, Robiola, Philadelphia and Scamorza, with values up to 0.4 mg g^{-1} fat. These data improved the knowledge about CLA content of dairy products, and this could make an accurate estimate of CLA ingested by Italian consumers.

1. Introduction

Conjugated linoleic acid (CLA) is the collective name for a group of linoleic acid isomers with conjugated double bonds. The main health-benefical CLA isomers are

the *cis9,trans11* (*c9,t11*) and the *trans10,cis12* (*t10,c12*), with the first playing a protecting role against cancer and atherosclerosis (Bhattacharya et al., 2006) and attenuating insulin resistance (Taylor & Zahradka, 2004), whereas the latter are related to the increase in energy expenditure and fat oxidation, decrease of adipocyte size and inhibition of some enzymes of fatty acid metabolism and lipogenesis (Bhattacharya et al., 2006). As reported by Ip, Singh, Thompson and Scimeca (1994), a recommended daily CLA intake of 3 g day^{-1} was recommended to reduce cancer risk. Anyway the authors did not specify to which isomer the recommendation referred. Successively, Ip et al. (1999) concluded that the main effect in reducing the mammary cancer risk could be imputed to the *c9,t11* isomer.

One of the main sources of CLA for humans is milk and dairy products (i.e., yogurt, fermented milk, soft and hard cheeses, etc.). It is well know the main factor affecting final CLA content of milk is animal feeding strategy. In particular, milk from lactating dairy cows fed fresh forage has a higher CLA content than milk from silage-fed cows (Chilliard & Ferlay, 2004). Otherwise, the milk processing and storage conditions generally do not affect the CLA concentration of milk fat (Bisig, Eberhard, Collomb, & Rehberger, 2007).

To provide information about CLA content of different dairy products available for consumers, different works were previously published by our research groups (Prandini et al., 2001; Prandini, Sigolo, Cerioli, & Piva, 2009; Prandini, Sigolo, & Piva, 2009; Prandini, Sigolo, & Piva, 2011; Prandini, Sigolo, Tansini, Brogna, & Piva, 2007). Nevertheless, not one complete database about CLA content in commonly marketed fresh and ripened cheeses is currently available to our knowledge. Thus, the aim of the present work was to complete the data obtained in previous studies with a survey on the *c9,t11* and *t10,c12* CLA content in cheese typologies collected from Italian large-scale retail trade (LRT) and not still characterized for these parameters. This is an integrative part of a extend work, studying the CLA content in foods, with the objective of estimating the daily CLA intake in an Italian cohort.

2. Materials and Methods

2.1 Sampling

A total of 102 samples of cows' milk cheese (except Feta) were collected from Italian LRT during a one-year period, from January to December 2011 to cover the

possibilities of consumer purchase. Cheese produced with milk from the spring and summer period by pasture-fed animals was avoided, in order to reduce CLA variation due to the animal diet. For each sampled cheese, the evaluation was based on ripening period and on information provided on the label (milk species, microbial starters, shelf life).

2.2 Chemical analysis

The moisture, fat, protein (total nitrogen x 6.38) and carbohydrate contents were determined in accordance with International Dairy Federation (IDF) 4:2004, 221:1998, 20-1:2001 and 79-2:2002 methods, respectively.

The lipid extraction was in agreement to Folch's technique (Christie, 1989) as modified by Prandini et al. (2007). Then, two 100-mg quantities (fat samples A and B) were taken from the fat extracted for every cheese sample and transferred in two different glass-stoppered test-tube of approximately 10 mL capacity. The preparation of *c9,t11* and *t10,c12* methyl esters was conducted in accordance with the method described by Prandini et al. (2007) on the fat samples A by using an internal standard (IS, nonadecanoate methyl ester acid, 0.3 mg mL^{-1}; Sigma-Aldrich Inc. Pleasant Gap, PA, USA) and on the fat samples B without addition of IS. *c9,t11* and *t10,c12* methyl esters were quantified using a GC (Varian 430) equipped with a flame ionization detector and a CP-Select CB capillary column for fatty acid methyl esters (100 m* 0.25 mm i.d.; 0.25 mm i.d; 0.25 μm film thickness; Chrompack, Varian, Inc., Palo Alto, CA, USA). GC oven parameters and gas variables were: isotherm analysis at 175°C for 65 min; temperature of injection and detector 250°C; injection volume 1μL; gas carrier He and its flow rate 1.5 mL min^{-1}. *c9,t11* and *t10,c12* isomer peaks were identified by comparison with the retention times of reference standards (methyl *c9,t11* and *t10,c12* octadecadienoate; Matreya, Pleasant Gap, PA, USA). A peak overlapping was found for C19:0 (IS), thus the area of the interfering peak was measured injecting each sample with and without IS; this area was then subtracted from C19:0 area to calculate the *c9,t11* and *t10,c12* contents in the cheese samples. The *c9,t11* and *t10,c12* levels were expressed in mg g^{-1} of fat.

In our study, the co-elution of the *c9,t11+t7,c9+t8,c10* triplet (Blasko et al., 2009) was not checked owing to commercial unavailability of the *t7,c9* and *t8,c10* isomer standards. Nevertheless, these isomers occur in very low amounts in milk fat (Kraft,

Collomb, Mockel, Sieber, & Jahreis, 2003). Thus, in our work the hypothetical *c9,t11+t7,c9+t8,c10* peak was easily named *c9,t11* peak.

2.5 Statistical analysis

Data were analyzed using parametric one-way ANOVA by GLM procedure of SAS (version 9.3, 2010), except for non-normal distributed CLA *t10,c12*, that were analyzed using Kruskal–Wallis non parametric ANOVA. The fixed tested effects were cheese type (n=16), curd cooking (n=3), texture (n=3) and ripening time (n=4). The least significant difference was generated from Tukey's test and it was used as the basis of the multiple comparisons among means, except for CLA *t10,c12* means that were compared with Behrens Fischer non parametric multiple comparison test. The significance level was set as $P < 0.05$.

3. Results and Discussion

From a nutritional point of view, improving the knowledge on CLA content of foods, in particular dairy products, could improve the estimate of CLA ingested by consumers. Furthermore, this could be useful to reduce the gap between RDI and effective amount of CLA assumed by humans, providing revised nutritional guidelines. The present study was based on a random sampling of main cheeses sold by Italian LRT and it was not possible to obtain any information about CLA content of the original milk.

Table 1 displays the chemical composition and *c9,t11* and *t10,c12* CLA isomer contents. As expected, fresh cheeses (Belpaese, Stracchino, Robiola, cows' milk Ricotta, Feta, Crescenza, Philadelphia) contained more moisture ($P<0.05$) and consequently less fat ($P<0.05$) and protein ($P<0.05$) than ripened cheese.

Gruyere and Feta had the highest *c9,t11* contents ($P<0.05$). The high *c9,t11* level in Gruyere could be due either to the ripening period or the use of *Propionibacterium spp.* starters. Kim et al. (2009) reported that long-ripened cheeses contain more CLA than others. Accordingly, the highest ($P<0.05$) *c9,t11* CLA isomer level was observed in long-ripening cheeses (>180 days, table 2). Furthermore, Gruyere contains *Propionibacterium spp.*, a starter already known as CLA promoter (Jiang, Bjorck, & Fonden, 1998). Sieber, Collomb, Aeschlimann, Jelen, and Eyer (2004) reviewed the impact of microbial cultures on CLA content, suggesting that CLA is produced from linoleic acid through the action of primary or secondary cultures used in cheese

processing. In particular, Jiang et al. (1998) reported the *Propionibacteria spp*. ability to convert up to 90% of free linoleic acid in total CLA (*c9,t11*; *t10,c12*; *t9,t11*; *t10,t12*). This finding was in accordance with those of Alonso, Cuesta and Gilliland (2003), Coakley et al. (2003), Kim and Liu (2002) and Kishino, Ogawa, Ando, Omura and Shimizu (2002) showing that defined strains of *Lactobacilli spp.*, *Bifidobacteria spp.* and *Propionibacteria spp*. were able to convert efficiently linoleic acid to CLA. However, in order to study the factors that might affect the *c9,t11* CLA level in cheeses, the whole production system should be carefully checked.

Feta is a typical Greek cheese made from ewes' milk or from a mixture of ewes' and goat milk (≤ 20%) (Salvadori del Prato, 2001) and this can explain the high *c9,t11* level found in Feta (Table 1) since ewe's milk has more CLA than cows' milk (Jahreis et al., 1999).

The *c9,t11* content of sampled cheeses (5.77±1.57 mg g^{-1} fat; range 4.32-10.21 mg g^{-1} fat) was similar to those obtained by Nunes and Torres (2010) and Jiang, Björk and Fondén (1997) in Brazilian and Swedish dairy products, respectively. Lower values were found by Lin, Boylston, Luedecke, and Shultz (1998), Ma, Wierzbicki, Field and Clandinin (1999) and Seckin, Gursoy, Kinik and Akbulut (2005) in US, Canadian and Turkish cheeses, respectively, whereas higher levels were reported for cheeses sampled in France (Lavillonniere, Martin, Bourgnoux and Sebedio, 1998), the Azores (Pestana et al., 2005) and Greece (Zlatanos, Laskaridis, Feist and Sagredos, 2002). Furthermore, *t10,c12* CLA isomer was almost absent, being detected only in Gruyere (Table 1) and in trace in other cheeses (i.e., Stracchino, Robiola, Philadelphia, Scamorza), with values ranging from 0.1 to 0.4 mg g^{-1} fat. Higher *t10,c12* CLA amounts were found in Greek and Canadian cheeses (Ma, Wierzbicki, Field and Clandinin, 1999; Zlatanos et al. 2002), while it was not detected in Azorean ones (Pestana et al., 2005).

4. Conclusion

Comparing our results with data from other countries, differences were found in *c9,t11* and *t10,c12* isomer contents, thus suggesting that CLA food data produced on a National scale should be provided to implement knowledge concerning the potential CLA intake used in epidemiological studies.

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Table 1. Average (± standard deviation) chemical composition (g/100g) and *c9,t11* and *t10,c12* CLA isomer concentrations (mg g^{-1} fat).

Cheese	n^a	Moisture	Fat	Protein	Carbohydrate	*c9,t11* CLA	*t10,c12* CLA
All samples	102						
Provola	6	43.89±6.01	27.08±4.47	24.55±1.82	2.00±0.20	6.96±5.12	n.d.
Provolone	6	40.02±2.14	30.33±1.47	24.48±1.71	2.00±0.15	4.92±0.23	n.d.
Caciotta	6	45.20±3.77	28.58±3.29	23.80±4.97	0.70±0.21	5.98±1.18	n.d.
Gouda	6	40.47±2.47	28.83±3.04	20.61±4.73	0.03±0.01	4.32±1.43	n.d.
Montasio	6	34.17±2.31	35.00±1.48	25.66±1.32	0.02±0.01	4.78±1.23	n.d.
Gruyere	6	38.30±0.89	34.92±0.92	27.44±0.87	0.50±0.02	10.21±2.47	0.40±0.63
Asiago	12	38.97±4.51	31.42±1.47	24.80±2.23	0.50±0.09	5.91±1.95	n.d.
Caciocavallo	6	36.70±2.14	29.50±2.21	31.77±2.42	2.30±0.23	5.35±2.77	n.d.
Belpaese	6	50.10±6.93	24.42±4.99	20.07±3.18	2.50±0.20	5.22±1.97	n.d.
Stracchino	6	56.86±2.92	25.33±3.61	12.93±3.58	2.40±0.19	5.01±1.26	0.14±0.33
Robiola	6	57.06±2.83	33.58±3.37	7.85±1.85	1.50±0.16	4.77±1.16	0.17±0.41
cows' Ricotta	6	75.37±3.24	10.67±1.94	8.53±2.27	3.50±0.28	5.53±1.08	n.d.
Feta	6	53.09±1.25	26.67±1.47	17.31±0.81	0.70±0.04	8.50±1.95	n.d.
Crescenza	6	60.49±4.97	21.67±6.70	15.64±1.66	1.80±0.17	4.76±0,79	n.d.
Philadelphia	6	65.93±8.13	22.29±10.59	8.68±1.75	3.10±0.21	5.41±1.15	0.16±0.38
Scamorza	6	51.45±2.26	20.83±2.07	22.60±1.33	0.50±0.03	4.93±0.31	0.16±0.39
√MSE[b]		4.100	4.069	4.220	1.654	2.025	0.238
P		<0.05	<0.05	<0.05	<0.05	<0.05	n.s.
LSD[c]		8.223	8.162	8.465	0.80	4.063	-

n.d., not detected; n.s., not significant.
n^a= number of samples.
√MSE[b] = root mean square error.
LSD[c]= least significant difference .

Table 2. Mean cheese *c9,t11* CLA isomer content (mg g^{-1} fat) considering manufacturing parameters (curds cooking, moisture content, ripening and seasoning).

	n^a	Mean ± SD	P	√MSE[b]	LSD[c]
Category	**102**				
Curds cooking					
Raw	48	5.77±2.40			
Semicooked	30	5.53±1.59	n.s.	2.391	-
Cooked	24	6.16±3.10			
Moisture Texture					
Hard	30	6.43±2.82			
Semi-hard	24	5.55±2.87	n.s.	2.366	-
Soft	48	5.52±1.69			
Ripening					
fresh (<45 days)	68	5.70±2.18			
medium (45<days<90)	15	4.85±1.92	<0.05	2.241	2.241
mid-long (90<days<180)	10	5.45±2.03			
long (>180 days)	9	8.45±3.28			

n.s., not significant.
n^a= number of samples.
√MSE[b] = root mean square error.
LSD[c]= least significant difference.

XI. MANUSCRIPT 3

In Press in Italian Journal of Animal Science

Contents of conjugated linoleic acid (CLA) isomers (*cis9,trans11* and *trans10,cis12*) in ruminant and non-ruminant meats available in the Italian market

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Abstract

Conjugated linoleic acid (CLA) isomers are considered healthy factors due to their anticarcinogenic and anti-atherosclerotic properties, as well as lipolytic effect. Recommended daily intakes of 3 g CLA/day/person has been proposed to obtain biological effects in humans. The aim of this work was to provide data on *cis9,trans11* (*c9,t11* CLA) and *trans10,cis12* (*t10,c12* CLA) contents in meats collected from Italian large-scale retail trade (LRT) and completing a food CLA database. In a first study, beef loin meats (n= 42), sampled from Italian markets, were characterized for some information available for consumers by labelling: origin (i.e., Ireland, France-Italy, Piedmont) and sex of animals. No differences were observed for *c9,t11* and *t10,c12* CLA contents of loin meat from male or female. Piedmontese meat showed lower ($P<0.05$) *c9,t11* CLA level than Irish and French-Italian meats, whereas similar *t10,c12* CLA contents were measured in Piedmontese, Irish and French-Italian meats. Successively, a total of 84 samples of meats from different animal species were

collected from Italian LRT and characterized for their contents in *c9,t11* and *t10,c12* CLA. They were: male beef (18), female beef (19), veal (15), lamb (6), pork (7), horse (6), belly beef (6) and canned beef meat (7). Lamb meat had the highest ($P<0.05$) *c9,t11* CLA content. The *c9,t11* CLA was lower than 2 mg/g fat in veal, pork and horse meats. Low *t10,c12* CLA amounts were found in all analyzed meat samples. These data provided information to estimate the average daily intake of CLA from meats in an Italian cohort which can be used in epidemiological studies.

Key words: *cis9,trans11* CLA, *trans10,cis12* CLA, Meat, Italian market, Animal origin.

Introduction

The term CLA refers to a group of conjugated isomers of linoleic acid which have been considered potent cancer preventive agents (Ip *et al*., 1994). Meat and dairy products are the main sources of CLA in human diet. CLA is produced by either ruminal biohydrogenation of dietary linoleic and linolenic acids to stearic acid or by endogenous synthesis from trans-vaccenic acid via Δ^9-desaturase (Griinari and Bauman, 1999; Griinari *et al*., 2000). Consequently, food sources derived from ruminants are significantly richer in CLA than those from monogastric animals (Schmid *et al*., 2006). CLA content in meat is affected by diet, animal and post-harvest related factors (Khanal and Olson, 2004; Dhiman *et al*., 2005). In particular, manipulation of animal diets can increase CLA content in meat, both from ruminant and non-ruminant. Feeding strategies, addressed to enhance CLA content in ruminant meat, are mainly based on supplementation of linoleic or linolenic acid as substrates for rumen biohydrogenation (Griswold *et al.*, 2003; Sackman *et al.*, 2003). Incorporation of CLA and trans-vaccenic acid in the animal diet could increase CLA concentration in non-ruminant meat (Glaser *et al*., 2002; Teschendorf *et al*., 2002). The CLA importance is related mainly to the healthy properties of two isomers, being *cis9,trans11* CLA (*c9,t11* CLA) and *trans10,cis12* CLA (*t10,c12* CLA) (Bhattacharya *et al*., 2006; Benjamin and Spener, 2009; Churruca *et al*., 2009; Stringer *et al*., 2010). Effects against mammary and prostate carcinogenesis, atherosclerosis (Bhattacharya *et al*., 2006) and insulin resistance (Taylor and Zahradka, 2004) have been attributed to *c9,t11* CLA. A recommended daily CLA intake of 3 g/d to reduce cancer risk was

reported by Ip *et al*. (1994). In a successive study, Ip *et al*. (1999) attributed main effect in reducing the mammary cancer risk to the *c9,t11* CLA isomer. Furthermore, the *t10,c12* CLA has been reported as CLA isomer responsible for improving features of the metabolic syndrome, such as hepatic steatosis, glucose intolerance and inflammation (Stringer *et al*., 2010). As a matter of fact, recommendation should be referred more properly to consumption of these isomers rather than all CLA isomers.

The aim of this work was to determine *c9,t11* and *t10,c12* CLA contents in meats available from Italian large-scale retail trade (LRT). In a first study (FS), the contents of these two CLA isomers in beef loin meats were related to different sex and origin of animals, on the base of meat label information. Then, a survey study (SS) was carried out to assess *c9,t11* and *t10,c12* CLA contents and chemical composition in meats from different animal species (ruminant and non-ruminant). The current studies represented an integrative part of an extended work conducted for studying the *c9,t11* and *t10,c12* CLA contents in foods (Prandini *et al*., 2001, 2007, 2009a, 2009b, 2011; Cicognini *et al*., 2013) and estimating the daily CLA intake in an Italian cohort, making them available for epidemiological studies.

Materials and Methods

Sampling

All the meat samples were purchased at supermarket in pre-wrapped food trays during a one-year period (from January to December 2011).

In FS study, male and female beef loin meats of three different origins (Piedmont, Ireland and French-Italy) were collected from Italian LRT. In particular, 14 samples were analyzed for each origin, of which 7 were female (heifer beef) and 7 male, for a total of 42 samples.

In SS survey, a total of 84 samples of ruminant and non-ruminant meat were collected from Italian LRT as follows: male beef (steers; 18); female beef (19); lamb (6); belly beef (6); veal (15); pork (7); horse (6); canned beef meat (7).

Chemical analysis

All meat samples were ground immediately after purchase with a mincing machine "La Moulinette 750 W" (Moulinex, France). The samples were then vacuum packaged and stored at -18°C and defrosted at room temperature before analysis.

The moisture was determined in accordance with the UNI ISO 1442:2010 method (ISO, 2010). Fat content was determined with the UNI ISO 1443:1991 method (ISO, 1991). Protein content was measured in accordance with the UNI ISO 937:1991 method (ISO, 1991). A N-protein conversion factor of 6.25 was used.

The lipid extraction was performed according to Folch's technique (Christie, 1989) as modified by Prandini *et al*. (2007). The preparation of *c9,t11* and *t10,c12* CLA methyl esters was conducted in accordance with the method described by Prandini *et al*. (2007) on two 100-mg amounts of fat extracted (with and without addition of internal standard; IS, nonadecanoate methyl ester acid, 0.3 mg/mL; Sigma-Aldrich Inc. Pleasant Gap, PA, USA) for each meat sample. The *c9,t11* and *t10,c12* CLA methyl esters quantification was performed by using a Varian 430-GC equipped with a flame ionization detector and a CP-Select CB capillary column for fatty acid methyl esters (100 m* 0.25 mm i.d.; 0.25 mm i.d; 0.25 μm film thickness; Chrompack, Varian, Inc., Palo Alto, CA, USA). GC oven parameters, gas variables and peak identification were as previously described (Cicognini *et al*., 2013). Since a peak overlapping was found for C 19:0 (IS), the area of interfering peak was measured running each sample twice, with and without IS. This area was then subtracted from C 19:0 area to calculate the *c9,t11* and *t10,c12* CLA contents (expressed in mg/g of fat) in the meat samples. In our study, the co-elution of the *c9,t11+t7,c9+t8,c10* triplet (Blasko *et al*., 2009) was not checked owing to commercial unavailability of the *t7,c9* and *t8,c10* isomer standards. Nevertheless, these isomers occur in very low amounts in ruminant fat (Kraft *et al*., 2003). Thus, in this work the hypothetical *c9,t11+t7,c9+t8,c10* peak was easily defined as *c9,t11* CLA peak.

Statistical analysis

Data were tested for normality with the Shapiro-Wilk test. Non-normal variables were log-normal transformed before statistical analysis (Petrie and Watson, 2006). Data of FS study were analyzed by using two way analysis of variance (ANOVA) by GLM procedure of SAS (2010) and fixed effects of the model were sex (i.e., male *vs.* female), origin (i.e., Ireland *vs.* France-Italy *vs.* Piedmont) and their first order interaction. Data of SS survey were analyzed by using one way analysis of variance (ANOVA) by GLM procedure of SAS (2010) and fixed effect of the model was meat types (n=8). The significance level was set at $P<0.05$.

Results and Discussion

FS study

Recently, the main LRT located on Italy provide a commercial meat differentiation based on origin of carcasses. In particular, the main product typologies could be grouped as follows: Irish, French-Italian and Piedmontese meats. Piedmontese is the most important Italian autochthonous beef breed contributing for 37% to the beef production and for approximately 50% to the gross saleable product in Piedmont (Associazione Nazionale Allevatori dei Bovini di Razza Piemontese, 2011; Brugiapaglia *et al*., 2013). The Irish meat refers to meat obtained from animals either born or produced in Ireland, whereas the French-Italian to meat obtained from animals born and grown for a period in France, and then grown-finished and slaughtered in Italy. The other beef meats could be considered of less importance because of their lower market share and then were not considered in our current study (Rama *et al*., 2013). The animal sex is another information provided by labelling for loin cut which is one of the meat cuts with higher share. Based on this differentiation, this study had the object to verify differences in *c9,t11* and *t10,c12* CLA contents of beef loin meats from animal of different origin and sex.

Table 1 shows the *c9,t11* and *t10,c12* CLA contents and chemical composition of analysed beef loin meats. No differences were found in the *c9,t11* and *t10,c12* CLA contents of loin meat from male and female animals. Instead, both fat, moisture and protein contents differed ($P<0.05$). Female loin meat was 58% richer ($P<0.05$) in fat than male loin meat, therefore a portion of female loin meat might contain twice as much the *c9,t11* CLA content than the same portion of male loin meat.

Loin meats from Ireland, France-Italy and Piedmont showed differences in chemical composition. In particular, Piedmontese meat showed lower ($P<0.05$) content of intramuscular fat (-68% on average) compared with Irish and French-Italian meats. The Piedmontese breed is characterized by muscle hypertrophy or double-muscled. In agreement with our result, this characteristic was associated with meat with lower fat content than meat from normal animals (Aldai *et al*., 2006). Piedmontese meat exhibited also lower ($P<0.05$) *c9,t11* CLA level (-35% on average) than Irish and French-Italian ones. Differences in fat content and a possible lower activity of Δ^9 desaturase enzyme in leaner animals, as reported by Siebert *et al*. (2003), Aldai *et al*.

(2006) and Brugiapaglia *et al*. (2013), could explain the lower *c9,t11* CLA content in leaner animals compared with fatter animals. Moreover, a high positive correlation between *c9,t11* CLA and intramuscular fat content was reported by Brugiapaglia *et al*. (2013). No difference was instead found in *t10,c12* CLA content among the Piedmontese, Irish and French-Italian meats. On the other hand, the ruminal biohydrogenation is the only synthesis pathway responsible for the *t10,c12* CLA level in ruminant products as animal tissues do not possess the desaturase enzyme capable of inserting a C 12-double bond into the *t*10 C18:1 molecule (Raes *et al*., 2004).

SS survey

To our knowledge, little information (Mele *et al*., 2008; Serra *et al*., 2009; Brugiapaglia *et al*., 2012) are currently available to estimate the consumption of *c9,t11* and *t10,c12* CLA from meat in Italian consumers. The aim of current survey was to provide *c9,t11* and *t10,c12* CLA values of the main meat typologies commercialized in Italy and, on the basis of other works conducted by this research group on other foods, to estimate the daily *c9,t11* and *t10,c12* CLA intake in Italy.

Table 2 shows the *c9,t11* and *t10,c12* CLA contents and chemical composition of meats from different animal species. Lamb meat had the highest ($P<0.05$) *c9,t11* CLA content. In agreement, a review of Schmid *et al*. (2006) reported that *c9,t11* CLA content in lamb meat could range from 4.3 to 19.0 mg/g fat. High *c9,t11* CLA content of lamb meat could be associated with diet, being lamb fed with sheep milk that is recognized as the richest milk in CLA of all species (Jahreis *et al*. 1999). With values numerically higher than 11%, lamb and belly beef showed high fat contents. In agreement with our results, higher intramuscular fat levels were associated with higher *c9,t11* CLA contents, being this CLA isomer predominantly deposited in the triacylglycerols (Lorenz *et al*. 2002; Raes *et al*., 2004). The *c9,t11* CLA was lower than 2 mg/g fat in veal, pork and horse meats. Veal is cow's milk fed. Thus, *c9,t11* CLA amount in veal tissues derives essentially from the animal diet being cow's milk poor in polyunsaturated fatty acids precursors of the *c9,t11* CLA synthesis in the rumen. Being non-ruminant animals, pork and horse meats showed *c9,t11* CLA levels lower than 1 mg/g, in agreement with previous studies (Chin et al., 1992; Dufey, 1999; Schmid *et al*., 2006). Studies carried out on mice have shown that CLA is synthetized endogenously from dietary trans-vaccenic acid (Santora *et al*., 2000; Banni *et al*., 2001; Khanal and Dhiman, 2004). Synthesis of CLA from trans-vaccenic acid has been

shown to occur in humans too (Adolf *et al*., 2000). Moreover, Alonso *et al*. (2003) and Coakley *et al.* (2003) reported that several species of bacteria derived from the human intestine can synthesize CLA. Canned beef meat showed a *c9,t11* CLA content similar to that found in raw beef meat both from male and female animals.

Low *t10,c12* CLA amounts were found in all analyzed meat samples. On the other hand, as reported above, *t10,c12* CLA is produced only by ruminal biohydrogenation (Raes *et al*., 2004). Beef female meat had the lowest ($P<0.05$) *t10,c12* CLA level together with non-ruminant meats (pork and horse), whereas beef male and veal meats showed intermediate *t10,c12* CLA values ($P<0.05$). Belly beef showed the highest ($P<0.05$) level of *t10,c12* CLA. Since in belly beef most of fat is subcutaneous, the high *t10,c12* CLA level found in this cut of beef meat might suggest that this CLA isomer is deposited mainly in subcutaneous fat, rather than in intramuscular fat.

Conclusions

This study is an integrative part of an extended work studying the CLA content in foods to estimate the daily CLA intake in an Italian cohort. In particular, it provided information on the *c9,t11* and *t10,c12* CLA contents in meats available from Italian LRT. Generally, meat contains low amounts of *c9,t11* CLA and almost negligible levels of *t10,c12* CLA for appreciation of health benefits in humans. Consequently, the consumption of other foods, such as milk and their by-products, should be encouraged to improve daily CLA intake by humans. Alternatively, specific feeding strategies should be taken in account in order to enhance the *c9,t11* and *t10,c12* CLA contents in meat.

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Table 1. Average *c9,t11* and *t10,c12* CLA contents (mg/g fat) and chemical composition (%) of beef loin meat from animals of different sex and origin.

	Sex[1]		Origin[1]			
	Male	Female	Ireland	France-Italy	Piedmont	√MSE[3]
CLA *c9,t11*	2.98	2.88	3.34[a]	3.40[a]	2.19[b]	1.242
CLA *t10,c12*[2]	0.04	0.01	0.01	0.03	0.04	0.461
Fat[2]	2.54[b]	5.98[a]	4.46[a]	6.70[a]	1.80[b]	0.687
Moisture	74.21[a]	71.19[b]	72.26[b]	70.58[b]	75.00[a]	2.488
Protein[2]	23.18[a]	22.03[b]	22.36	22.13	23.21	0.061

[1]The interaction of sex and origin was not statistically significant; [2]values were log-transformed before statistical analysis; [3]root of mean-square error (MSE); [a,b]means in the same row within sex and origin with different superscripts differ (P<0.05).

Table 2. Average *c9,t11* and *t10,c12* CLA contents (mg/g fat) and chemical composition (%) of meats from different animal species.

	All	Beef male	Beef female	Veal	Lamb	Pork	Horse	Belly beef	Canned beef meat	√MSE[2]	P of the model
n	84	18	19	15	6	7	6	6	7		
c9,t11 CLA		2.98^{bc}	2.88^{bc}	1.28^{c}	9.84^{a}	0.67^{c}	0.34^{c}	4.24^{b}	2.01^{bc}	1.747	< 0.05
t10,c12 CLA[1]		0.04^{b}	0.01^{c}	0.07^{b}	0.02^{bc}	0.01^{c}	0.01^{c}	0.36^{a}	0.01^{c}	0.822	< 0.05
Fat[1]		2.54^{c}	5.98^{bc}	5.15^{bc}	11.59^{ab}	4.80^{bc}	2.73^{c}	19.81^{a}	1.35^{c}	0.810	< 0.05
Moisture		74.21^{b}	71.19^{b}	73.11^{b}	68.62^{b}	70.80^{b}	72.67^{b}	55.35^{c}	81.78^{a}	3.761	< 0.05
Protein[1]		23.18^{a}	22.04^{ab}	21.83^{ab}	19.80^{b}	22.73^{ab}	23.00^{a}	16.55^{c}	13.63^{d}	0.095	< 0.05

n, number of samples; [1]values were log-transformed before statistical analysis; [2]root of mean-square error (MSE); [a,b,c,d]means in the same row with different superscripts differ (P<0.05)

XII. MANUSCRIPT 4

For Public Heatlh and Nutrition

Estimation of *c9,t11* and *t10,c12* conjugated linoleic acid isomers intake in a cohort of healthy students in Italy

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Abstract

During the last two decades, a lot of studies have been done to investigate the healthy properties of conjugated linoleic acid (CLA) isomers. However few works discussed the actual CLA intake in humans.

To investigate the dietary behaviour of consumers, a three-days food questionnaire was administered to a cohort of 27 Italian students. Data from the food diaries were combined with a data-base on CLA content in foods. When a food was not included,

a sample of it was analysed for its content in both *cis9,trans11* and *trans10,cis12* CLA.

CLA intake ranged from 8.7 mg/day to 545.4 mg/day and was higher in male than in female (164.70 mg/day *vs* 96.70 mg/day).

The estimated CLA intakes in Italy were compared with data obtained in studies from other countries.

Keywords: CLA intake; food diary; CLA database; dairy products; meat products

Abbreviations: CLA, conjugated linoleic acid; LA, linoleic acid; FAME, fatty acid methyl esters; *t10,c12*, *trans10,cis12 CLA; c9,t11, cis9,trans11 CLA.*

1. Introduction

In the last two decades *cis9,trans11* and *trans10,cis12* conjugated linoleic acid isomers were recognised as healthy factors (Bhattacharya *et al.*[1]; Park *et al.*[2]; Raff *et al.*[3]). CLAs are naturally present in ruminant food products as intermediates in the biohydrogenation of linoleic acid by rumen bacteria. *c9,t11* isomer is also produced by vaccenic acid desaturation in the mammary gland (Bauman *et al.*[4]; Griinari and Bauman[5]), covering from 75 to 90% of total CLA in milk (Bauman *et al.*[6]). According to animal and cell culture experiments, *c9,t11* plays a protective role against cancer and atherosclerosis (Bhattacharya *et al.*[1]) and can also attenuate insulin resistance (Taylor and Zahradka[7]; Hong *et al.*[8]). *t10,c12* isomer can be instead related to the increase in energy expenditure and fat oxidation, to the decrease of adipocyte size and the inhibition of some enzymes involved in fatty acid metabolism and lipogenesis (Blankson *et al.*[9]; Bhattacharya *et al.*[1]). Due to these healthy properties, CLA content in food and the factors affecting CLA levels were also investigated. CLA content in milk and meat depends mainly on the feed regimen: as a matter of fact pasture was reported as a CLA enhancing factor, due to its high polyunsaturated fatty acid (PUFA) content, that are CLA precursors (Kelly *et al.*[10]; Collomb *et al.*[11]). Also species, breed, age and individual conditions could influence CLA content. As a matter of fact ewe milk is the richest in CLA among all milk types (Jahreis *et al.*[12]). Moreover CLA content in food can be affected by the food manufacturing: for example cooking temperatures, ripening period and microbial

starter selection could influence the final CLA amount (Kim *et al*[13], Sieber *et al*[14]). Several studies were performed to estimate the CLA daily intake in human subjects from different countries, and a strong variation in CLA intake among different countries was found. In Germany Fritsche and Steinhart[15] using data from a national dietary survey estimated a daily CLA intake in man and women respectively of 440 and 360 mg/d. In the same year Ritzenthaler *et al.*[16] published US data from 3-days dietary records and calculated a CLA intake of 104 mg/d CLA for women and 176 mg/d for men. The variation in daily intake among different countries may be due to dietary behavior and preferences, food processing, animal feeding. However also the method used to estimate the food intake could explain these discrepancies.
Based on recent anticancer researches, *Ip et al.*[17] proposed 3g/day as the lowest daily CLA intake to obtain anti-carcinogenic effects. Most of the studies on CLA intake however reported intake very far from this value. To our knowledge, no comprehensive information about CLA intake in Italy are nowadays available.
Thus the main aim of our work was the estimation of the daily *c9,t11* and *t10,c12* intake of young people in Italy. The data on CLA content in foods were reported in previous works (Prandini *et al*[18,19], Cicognini *et al*[20,21]) and applied to a 3-days food diary record.

2. Materials and methods

2.1 Subjects and study design

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, using the food record model. Written informed consent was obtained from all participants. 27 healthy subjects were recruited using advertisements posted on the university showcase. Subjects were selected basing on age, medical conditions and dietary behaviour. The selected age ranged from 19 to 25 years old. Individuals with dyslipidemia, pregnant or breast-feeding and consuming an energy-restricted diet were excluded. Vegan or vegetarian people were not considered in this study. Subjects who satisfied inclusion criteria were then given instructions to write off a 3-days food diary previously validated and used in the Italian National Survey INRAN-SCAI (Leclercq *et al.*[22]) that was given them after they had signed the consent. They were asked to eat as usual in subsequent 3 days to emulate as best their habits. They were educated to indicate clearly each food they

introduced, when and where they ate it, and the number and quantity of serving portions. If they were not able to weight exactly each portion, a photographic atlas from EPIC study (Pisani *et al.*[23]) and Moli-Sani Project (Iacoviello *et al.*[24]) was used. In this atlas, plates, cup and spoons were illustrated in three portion sizes (small, medium and large): subjects had to choose the most similar image to their portion. At the end of the three days, food diaries were checked to study the daily CLAs intake. For each subject the mean daily intake of *c9,t11* and *t10,c12* were estimated by determining the content of these isomers in consumed foods over the weighted period.

2.2 Database of CLA content of food

Dairy products and meat products: data were obtained from our previous works (Prandini *et al.*[18,19], and Cicognini *et al.*[20,21]). Grana Padano (GP) and Parmigiano Reggiano (PR) were discussed toghether. Subjects did not specify which type of Grana cheese was consumed, so an average of the CLA content of the two cheeses was used (6.0 mg *c9,t11*/g fat).

Confectionary products were purchased in September 2012 in North-Italian large-scale retail trade. They included the main commercial snacks available in Italian market. In table 1, 2 and 3 *c9,t11* and *t10,c12* amounts in foods were showed.

2.3 Sample preparation

Each hard and semi-hard sample was ground immediately after purchase with a mincing machine “La Moulinette 750 W”(Moulinex, France), while soft cheeses and cakes were gently hand pressed. The samples were then stored at -18°C and defrosted at room temperature before analysis.

2.4 Reagents

Chloroform, metanhol and sodium sulfate anhydrous were purchased from Carlo Erba Reagenti SpA (strada Rivoltana, Rodano, Milano, Italy). The standards *c9,t11*-octadecadienoic acid and *t10,c12*-octadecadienoic acid were purchased by Matreya (Superchrom S.r.l., Milan, Italy); methyl-nonedecanoate (C19) standard was purchased by Sigma Inc. (Sigma-Aldrich Co, Milan, Italy).

2.5 Chemical analysis

2.5.1 CLA quantification

Lipid extraction was performed according to the modified Folch's technique (Christie[25]): 10 g cheese or 30 g meat were mixed respectively with 100 or 300 ml chloroform–methanol mixture (2:1, v/v); after homogenizing in a Ultra-Turrax T25 homogeniser (Janke & Kunkel, GmbH & Co, Staufen, Germany), the mixture was agitated for 60 min and was then filtered into a separator funnel through filter paper (Albet folded circles, 130 cm, extra rapid). Seventy-five ml of saturated NaCl solution were added to the filtrate; chloroform phase was subsequently recovered, dehydrated with anhydrous sodium sulfate (Na_2SO_4) and dried with a rotary evaporator at 40 °C under vacuum.

Preparation of CLA isomers methyl esters: the esterification was in accordance with the method described by Bannon *et al.*[26] with some differences. One hundred milligrams of extracted fat were weighed in a glass-stoppered test-tube of approximately 10 ml capacity and dissolved in 2 ml of hexane solution containing an internal standard (nonadecanoate methyl ester acid, 0.3 mg/ml). 2N methanolic-potassium-hydroxide (100 µl) was added, the mixture was shaken vigorously for 30 s and allowed to react for a total of 6 min at room temperature (ca. 208°C). The catalyst (KOH) was neutralised immediately by adding 2N hydrochloric acid (100µl) with shaking, to the methyl orange end-point. The hexane phase, containing the CLA methyl esters (FAME), was separated by centrifugation and subjected to gas chromatography.

Gas-chromatographic analysis: CLA methyl esters were quantified using a GC (Varian 430) equipped with a flame ionisation detector and a CP-Select CB capillary column for FAME (100 m* 0.25 mm i.d.; 0.25 mm i.d; 0.25 µm film thickness; Chrompack, Varian, Inc., CA). GC oven parameters and gas variables: isotherm analysis at 175°C for 65min, temperature of injection 250°C, detector temperature 250°C, injection volume 1µl, gas carrier He and its flood 1.5 ml/min. CLA isomer peaks were identified by comparison with the retention times of reference standards adequately methylated (*c9,t11* and *t10,c12* octadecadienoic acid): C19:0 (IS) was eluted at 25.86 min, C18:2 *c9,t11* at 33.72 min and C18:2 *t10,c12* at 34.77 min. CLA content, expressed in mg/g fat, was determined by the following formula:

*CLA(mg/g fat)=(area CLA*conc IS*CFCLA)/(areaIS*conc.fat*1.04)*

where ‘‘1.04’’ is the conversion factor from methyl ester to fatty acid and ‘‘CF’’ the isomer correction factor obtained from an average of 10 injections of a mixture containing 0.025g standard isomer (*c9,t11* or *t10,c12*) adequately methylated and 0.010 g nonadecanoate methyl ester. The CF correction factor was determined as follows:

CF_{CLA}=(area $_{STD}$/area $_{CLA}$)*(conc $_{CLA}$/conc $_{STD}$)

2.5.2 Statistical analysis

CLA content of foods

All statiscal analysis were performed using the software SAS 9.2 (SAS Inst., Cary, NC, USA).

Data were tested for normality using the Shapiro-Wilk test, non-normally distributed data were log-transformed and, if log-transformed data were normally distributed, means were compared (ANOVA) by PROC GLM of SAS 9.2. Data related to CLA *t10, c12* were analyzed with a non-parametric test: Krauskal-Wallis (ANOVA).

The least significant difference (LSD) was obtained by Tukey’s test and used for the multiple comparison between means.

Significant level of difference was set al $P<0.05$.

Estimation of CLA intake

The comparison of means of CLA intake in males and females was carried out with the t Student test, while the multiple comparison between means was performed using the Bonferroni test.

3 Results and Discussion

3.1 Anthropometric measurements of volunteers

The mean BMI of female volunteers was 22.5 ± 3.6, whit a body fat percentage of 30.0 ± 5.6 % while the BMI value for male was 22.5 ± 1.7 and body fat percentage was 16.0 + 3.4 %.

3.2 CLA content in dairy products

CLA isomer amounts in dairy products were showed in Table 1.

c9,t11 is predominant among the CLA isomers in dairy products, while *t10,c12* is almost absent.

As expected, ripened cheeses contained more *c9,t11* than fresh cheeses (Fontina 8.1 mg/g fat; Emmental 7.66 mg/g fat) (Prandini *et al.*[18]). These values are higher than *c9,t11* content in mature Cheddar reported by Mushtaq *et al.*[27] in UK and by Chin *et al.* in US (respectively 6.1 and 4.7 mg/g fat). The high level of CLA in these cheeses could be related to the ripening period as well as the pasture feeding: Kim *et al.*[13] and Sieber *et al.*[14] reported that a medium-long ripening period increased CLA amount in cheeses. Moreover *Propionibacterium spp.* starter (Disciplinare di produzione della DOP "Fontina"[29]) could have affected CLA amount in Fontina DOP. As a matter of fact Sieber *et al.*[14] showed that CLA can be produced from linoleic acid through the action of *Lactobacillus spp.*, *Lactococcus spp.* and *Streptococcus spp.*, able to convert up to 10% of free linoleic acid in CLA, and of *Propionibacteria spp.* converting up to 90% of linoleic acid.

Butter content of *c9,t11* was higher than the estimation of Mushtaq *et al.*[27] (2.5 mg/g fat), Martins *et al.*[30] (3.87 mg/g fat), Chin et *al.*[31] and Ma *et al.*[32] (4.7 mg/g fat), while it was lower than German butter (9.4 mg/g fat) (Fritsche and Steinhart[15])

Sliced processed cheese contained 6.37 mg *c9,t11*/g fat, less than 11.9 and 7.3 mg/g fat found respectively in German and UK processed cheese and more than 4.65 mg/g fat reported in US work.

In dairy products *t10,c12* CLA was almost absent. It was found only in traces in Philadelphia and Scamorza (respectively 0,15 and 0,16 mg/g fat). Martins *et al.*[30] reported instead lower but detectable values: 0.03 mg/g fat in butter and 0.0004 mg/g fat in cheeses.

3.3 CLA content in meat

c9,t11 was the main CLA isomer also in meat products, while *t10,c12* amount in meat was lower probably because *t10,c12* derive only from the rumen production, while tissues do not have the needed desaturase enzyme (Raes *et al.* [32]).

Lamb was found the *c9,t11* richest meat (8,95 mg/g fat). This is in according with Martins *et al.*[30], who reported a *c9,t11* content of 8.73 mg/g fat. A similar amount (8.2 mg/g fat) was detected also in lamb from UK (Mushtaq *et al.*[27]). Fritshe and Steinhart[15] reported instead a higher CLA concentration (12 mg/g fat). In the works of Haumann and Snell[33], Ivan *et al.* [34] and Schmid *et al.*[35] CLA values in lamb ranged from 2 to 19 mg/g fat. The high values of CLA in lamb were expected: as a matter of fact lamb is fed with ewe milk that is naturally rich in CLA. Beef (2,89 mg/g fat) followed lamb meat. Our *c9,t11* value was in according with Martins *et al.*[30] while was lower than the *c9,t11* amount found in beef from UK, Germany and US (Mushtaq *et al.* [27]; Fritshe and Steinhart[15], Chin *et al.*[28]).

In our work the most relevant amount of *t10,c12* was found in calf meat as 0,07 mg/g fat, while beef contained 0,03 mg/g fat. Martins *et al.*[30] and Mushtaq *et al.*[27] instead reported higher amount in beef (respectively 0,11 and 0,07 mg/g fat).

3.4 CLA content in confectionery

Confectionery products included snacks from the most spread labels in Italy. Chocolate bars with or without cereals and sponge cake, wafer, brioches, puddings and ice creams were studied. CLA isomers content was showed in table 3. Chocolate pudding and cream caramel contained more *c9,t11* than the other confectionaries, respectively 5,17 and 4,29 mg/g fat; brioches filled with cream followed (3,00 mg/g fat). The CLA content of these confectionery products is most likely to have been derived from the milk involved in their manufacture.

3.5 Estimation of the mean daily CLA intake

In our cohort the calculated average daily *c9,t11* intake was 133,14 mg. A significant difference in CLA intake between male and female was found (Tab. 5). Males introduced daily 164,70 mg of *c9,t11*, while females introduced 96,90 mg/d. It has to

be considered that the CLA isomers intake is related to the total food intake, usually higher in males than females. Food consumption was affected by sex: males introduced significantly higher amounts of *c9,t11* from cheese (115,70 mg/g fat) and ham (2,12 mg/g fat) than females (respectively 65,79 and 0,98 mg/g fat). CLA intakes from other foods were instead similar in the two sexes.
Table 4 reports data on the contribution of different food groups on CLA intake. Cheeses accounted for more than 65% of CLA intake and can be recognised as the main sources of CLA for young people. Milk is the second food for contribution to CLA intake (15.39 %), while yoghurt, meat and confectionery showed a minor contribution (respectively 45,13 and 42,21 mg/day).

CLA sources

The percentages of CLA introduced with each food on total CLA intake were also showed. CLA intake from cheese was found the 61,96% of total CLA introduced. Milk percentage (13,20 %) was lower, but significantly higher than the other foods. Dairy foods were the main sources of CLA in the paper of Ritzenthaler *et al.*[16] also. This data were in agreement with the paper of Jiang *et al*.[36] who reported a significant positive relationship between milk fat intake and CLA content of adipose tissue. Also in the work of Voorips *et al.*[37] the main sources of CLA were dairy foods, however in this paper, butter accounted for almost the 30% of CLA intake, while in our experiment butter was poorly consumed by our volunteers.

CLA intake

Values from several countries were showed in table 6. Our results were in accordance with Mushtak *et al.*[27] and Ens *et al.*[38], who found a mean daily CLA intake respectively of 97,5 and 94,9 mg. Both the works used a dietary record in a small cohort. Martins *et al.*[30] and Fritshe and Steinhart[11] used instead national dietary survey data: the first work reported a CLA daily intake lowers than ours, while the second one showed higher intakes (400 mg/d). Parodi *et al.*[39] indicated a very high CLA intake in Australia ranging from 500 to 1500 mg/day and evaluated individual's dietary preferences and seasonal factors affecting CLA in milk and ruminant fat. Ritzenhaler *et al.*[16] estimated a CLA intake ranging from 104 for females to 176 mg/day for males in USA population, values very close to our results.

These data suggest that CLA intake is very far from recommended daily intake proposed by Ip *et al.*[17] , however this gap is probably lower than expected because it has to be considered that 20% of the dietary vaccenic acid is converted endogenously to CLA in human subjects (Turpeinen *et al.*[40]). Dairy products contain about twofold vaccenic acid than CLA, thus the conversion factor to the real CLA intake can be calculated as 1,4.

Other factors explaining the difference in CLA intake between different Countries are related to factors affecting CLA levels in food, e.g. animal feeding (grazing increase CLA levels in milk and meat), animal species (milk and meat from sheep are richest in CLA than similar products obtained from bovine or goats).

The methods used for estimating food consumption and CLA intake, can also affect the results. National dietary analyse data from cohort bigger than ones used when food frequency questionnaires are used, these different approach could affect the estimation of CLA intake.

3.4.1 Comparison between male and female CLA intake

The differences in CLA intake between males and females are shown in Tab. 2. Male subjects in our study daily introduced a CLA amount of 164,70 mg, while females reached an intake of 96,90 mg. A US study on 3-days women dietary record made by Ritzenthaler *et al.*[16] reported a lower daily CLA intake of 50 mg/d. Fritshe and Steinhart[15] described instead a daily men and woman intake respectively of 440 and 360 mg, an higher amount than our results. Mushtaq *et al.*[27] reported a CLA intake lower than ours, either in male (126.7 mg/d) and female (68.3 mg/d).

In our experiment a significant difference was found in the CLA intake from cheese and ham: males introduced more CLA with these products (115,7 and 2,12 mg/d respectively from cheese and ham) than females (65,79 and 0,97 mg/d).

3. Conclusions

Data obtained in the present study show that CLA intake in a cohort of University Italian students is lower than value proposed by Ip *et al*[17] as biologically relevant. Within the cohort, male subjects introduced more CLA than females one. Dairy foods were the most relevant sources of CLA in both sexes. These data confirm that in order to improve CLA intake, the main focus should be on dairy foods whose CLA

content can be increase by means of microbial starter with CLA producing bacteria or a more widespread use of grazing for lactating animals.

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Conflict of interest
Authors have not conflict of interest.

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Table 1. Average CLA isomers content in dairy products.

Dairy products	*c9,t11* CLA		*t10,c12* CLA		Reference
	mg/g fat	*mg/100g prod.*	*mg/g fat*	*mg/100g prod.*	
Butter	6.71	564.24	n.d.	-	Prandini et al. 2001
Yoghurt	4.45*(4.00)		n.d.	-	Prandini et al. 2007
Grana	6.00*(5.40)		n.d.	-	Prandini et al. 2009
Mozzarella	6.26	99.66	n.d.	-	Cicognini et al., unpublished data
Ricotta	5.18	55.27	n.d.	-	Cicognini et al.
Scamorza	4.62	96.23	0.16	3.33	Cicognini et al.
Sottiletta	6.37		n.d.	-	Cicognini et al., unpublished data
Fontina	8.11*(7.30)		n.d.	-	Prandini et al. 2007
Crescenza	4.20	91.01	n.d.	-	Cicognini et al.
Philadelphia	5.20	115.91	0.15	3.34	Cicognini et al.
Zola	5.16*(4.64)	148.30	n.d.	-	Prandini et al. 2011
Emmental	7.66*(6.89)		n.d.	-	Prandini et al. 2007
Caciocavallo	5.24	154.58	n.d.	-	Cicognini et al.
Caciotta	5.55	158.62	n.d.	-	Cicognini et al.

* *c9,t11* values are adjusted as 90% of total CLA reported by these authors.

Table 2. Average CLA isomers content (mg/g fat) in meat products (Cicognini et al.)

Meat	***c9,t11* CLA**		***t10,c12* CLA**	
	mg/g fat	***mg/100g prod.***	***mg/g fat***	***mg/100g prod.***
Beef	2.87	12.22	0.03	0.13
Calf	1.28	6.59	0.07	0.36
Lamb	8.95	103.73	0.03	0.35
Pork	0.68	3.26	0.02	0.10
Horse	0.35	0.96	0.01	0.03
Canned meat	1.99	2.63	0.01	0.01
Raw ham	0.62	10.25	n.d.	-
Cooked Ham	0.52	3.70	n.d.	-
Bresaola	0.64	1.21	n.d.	-
Pancetta	0.60	27	n.d.	-
Mortadella	0.58	14.73	n.d.	-
Salame	0.51	14.54	n.d.	-
Speck	0.57	8.16	n.d.	-

Table 3. Average CLA isomers content (mg/g fat) in sweets.

Confectionary	***c9,t11* CLA**		***t10,c12* CLA**
	mg/g fat	***mg/100g prod.***	***mg/g fat***
Milk chocolate bar with cream and wafer	0.02		n.d.
Milk chocolate bar 1	1.18		n.d.
Milk chocolate bar 2	1.18		n.d.
Milk chocolate tab with cereals	0.99		n.d.
Sponge cake covered and filled with milk	1.18		n.d.
Wafer	n.d.		n.d.
Chocolate brioche	n.d.		n.d.
Plum Cake	0.17		n.d.
Chocolate sponge cake	0.06		n.d.
Cream brioches	3.00		n.d.
Vanilla pudding	4.29		n.d.
Chocolate pudding	5.17		n.d.
Cream caramel	5.17		n.d.
Ice cream	n.d.		n.d.

Table 4. Average of *c9,t11* daily intake (mg) and of *c9,t11* % (on total *c9,t11*) introduced with each food in a cohort of 40 students.

Food	**CLA[A]**	**CLA %[B]**
Cheese	85.76[a]	61.96[a]
Milk	15.39[b]	13.20[b]
Yoghurt	7.81[b]	7.51[c]
Confectionery	4.71[b]	3.17[c]
Meat	4.39[b]	3.46[c]
Ham	1.43[b]	1.16[c]
Olive oil	4.54[b]	3.61[c]
√MSE[C]	91.42	13.99
P	<0.0001	<0.0001

Means in the same column with different superscripts differ ($P<0.05$).
CLA[A] = CLA intake (mg/day) from each food;
CLA %[B] = % CLA introduced with each food calculated on total CLA intake (3days);
√MSE[C]= root mean square error

Table 5. Average of daily *c9,t11* intake and of *c9,t11* % (on total *c9,t11*) introduced with each food in male (n=16) and female (n=24) subjects; Average of the total *c9,t11* intake of male and female in 1 and 3 days.

Food	**CLA[A]**				**CLA %[B]**			
	M	*F*	√MSE[C]	P	*M*	*F*	√MSE[C]	P
Cheese	115.70[a]	65.79[b]	228.80	<0.05	66.13	59.19	28.37	n.s.
Milk	20.57	11.93	47.19	n.s.	16.83	10.78	14.75	n.s.
Yoghurt	9.97	6.37	30.39	n.s.	8.56	5.94	14.62	n.s.
Confectionery	7.33	2.96	33.48	n.s.	3.73	2.79	7.57	n.s.
Meat	7.35	2.42	26.42	n.s.	5.26	2.26	5.58	n.s.
Olive oil	1.66	6.45	31.75	n.s.	1.85	4.78	6.65	n.s.
Ham	2.12[a]	0.98[b]	4.97	<0.05	1.63	0.84	1.39	n.s.
CLA1d[D]	164.70[a]	96.90[b]	89.94	<0.05				
CLA3d[E]	494.12[a]	290.70[b]	269.82	<0.05				

Means in the same row with different superscripts differ ($P<0.05$).
CLA[A] = CLA intake (mg/day) from each food;
CLA %[B] = % CLA introduced with each food calculated on total CLA intake;
MSE[C]= root mean square error
CLA1d[D]= Average of total CLA intake (mg) in 1 day
CLA3d[E] =Average of total CLA intake (mg) in 3 days

Table 6. Average CLA intake (mg/d) estimated in studies from several countries.

Year	Country	Daily intake	Estimated isomer	Method	Authors
2010	UK	97.5	*c9,t11*	3d dietary record	Mushtak *et al*.[30]
2001	Canada	94.9	*c9,t11*	7d dietary record	Ens *et al.*[31]
2007	Portugal	73.7	*c9,t11*	National dietary survey	Martins *et al.*[32]
1998	Germany	400	*c9,t11*	7d dietary record	Fritshe &Steinhart [11]
1994	Australia	500-1500	Total CLA	-	Parodi *et al.*[33]
2001	USA	79-133	*c9,t11*	3d dietary record	Rizenthaler *et al.*[12]
2002	UE	250	*c9,t11*	milk intake	Wolff & Pricht
1999	Sweden	350-430	*c9,t11*	1d dietary record	Jiang et al. (1999)
1998	USA	127	*c9,t11*	3d dietary record	Herbel et al. (1998)

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For Lipids

Breed and dietary linseed and protected fish oil affected gene expression in *longissimus dorsi* muscle of beef.

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Abstract

The aim of the work was to investigate the effects of breed (Aberdeen Angus (AA) and Belgian Blue (BB)) and diet promoting conjugated linoleic acid (CLA) synthesis or supplementing n-3 PUFA) (ω3) on the expression of genes related to lipid and CLA metabolism. The genes investigated can be divided into those encoding: 1) lipogenic enzymes such as *ACC*, *FAS*, *SCD*, *Δ6D*, *Δ5D* and *ELOVL5*; 2) factors involved in lipid metabolism such as *AMPKα*, *ADIPOQ* and *GPR43*; 3) transcription factors including *SREBP1c*, *PPAR γ* and *α* and *STAT5*; 4) lipid storage-associated proteins such as *ADFP*; and 5) energy metabolism such as *GLUT4*.

AA and BB breeds were chosen due to their different rates of maturity.

ACC and *SCD* gene expression were higher in BB (*P*=0,013; *P*= 0,055), while *FAS* expression was higher in AA (*P*=0,007).

FAS and *SCD* expressions were increased by n-3 FA supplementation (*P*=0,006; *P*=0,074).

Finally an interaction between breed and dietary n-3:n-6 ratio was observed for *PPARα* (*P*= 0,006) and *ADIPOQ* (*P*=0,055). Thus, *PPARα* expression was reduced by n-3 FA in AA and increased in BB; *ADIPOQ* was found higher in AA and lower in BB with CLA.

Thus breed and diet could affect the gene expression of CLA-related genes, however more studies are needed to describe the underlying mechanisms.

Keywords: gene expression, CLA, diet, SCD, PPARs, n-3 PUFA, n-6 PUFA

Abbreviations: fatty acid (FA); saturated fatty acid (SFA); polyunsaturated fatty acid (PUFA); α-linolenic acid (ALA); linoleic acid (LNA); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA); conjugated linoleic acid (CLA); acetyl-CoA carboxylase (*ACC*); fatty acid synthase (*FAS*); stearoyl-CoA desaturase (*SCD*); delta-6-desaturase (*Δ6D*); delta-5-desaturase (*Δ5D*); fatty acid elongase 5 (*ELOVL5*); activated protein kinase (*AMPKα*); adiponectin (*ADIPOQ*); G protein-coupled receptor (*GPR43*); sterol regulatory element-binding protein1c (*SREBP1c*); peroxisome proliferator-activated receptor γ and α (*PPARγ*; *PPARα*); statin (*STAT5*); adipose differentiation-related protein (*ADFP*); glucose transporter 4 (*GLUT4*); Aberdeen Angus (AA); Belgian Blue (BB).

1. Introduction

In the last decade many studies have been conducted in order to improve its fatty acid (FA) composition. In fact, meat was considered as a source of saturated fatty acids (SFA) that could be implicated in some cancers and in coronary heart disease (1). However beef also provides polyunsaturated FA, that can be distinguished mainly into n-3 PUFA, produced from α-linolenic acid (ALA, C18:3), and n-6 PUFA, produced from linoleic acid (LNA, C18:2) (Williams, 2000). Among n-3 PUFA, eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) are recommended by the American Heart Association for reducing the risk for coronary heart disease (CHD) (3,4).

The ratio n-6:n-3 PUFA was reported as an important risk marker for cancers and coronary heart disease (5)

n-6 PUFA include the isomers of conjugated linoleic acid (CLA), where *cis9,trans11* and *trans10,cis12* CLA are the most important: *c9,t11* CLA is believed to have anti-carcinogenic and anti-atherosclerotic properties and to reduce insulin-resistance (6,7) while *t10,c12* is mainly related to a decrease of fat mass (8).

Animal breed, sex, age and diet are the main factors affecting FA composition of meat (9). French et al. (10) showed that pasture enhanced CLA concentration, increased the ratio of PUFA:SFA and improved the n-6:n-3 PUFA ratio, if compared with grass silage and concentrate feeding. Moreover the improvement was related to the duration of grazing (11). Also oil supplementation affects the fatty acids profile of beef: fish oil supplementation enriched n-3 PUFA (3) while sunflower oil enhanced the amount of CLA in meat (12). However it was also reported that an accumulation of vaccenic acid as CLA precursor in tissues did not increase CLA (4), thus suggesting the involvement of other mechanisms. The alteration of lipogenic gene expression due to diet could be the limiting step, modifying related protein expression or enzyme activities (13,14,15,16). For example dietary CLA was reported to affect the expression of some of the most important genes implicated in the lipid metabolism (17,18); Waters et al. (15) showed that dietary n-3 PUFA inhibits the genes encoding for CLA synthesis; Hiller et al. (19) reported a beneficial decrease of n-6:n-3 PUFA ratio due to the reduction of *ACC*, *FASN* and *Δ6D* gene expression.

Thus there is a rising interest in elucidating the factors affecting the n-6 *vs*. n-3 PUFA balance together with increasing CLA concentration in meat, as CLA improves it's nutritional value due to the related healthy properties. Moreover a CLA intake of 3g/day should be reached, obtaining the minimum value needed for positive health effects from the exptrapolation af animal diet fro humans (20). In addition more recent studies showed that an intake of 0.8-3 g/day could be sufficient to exert the CLA effects (21)

This work aimed to investigate the influence of breed and of the n-3 *vs*. n-6 PUFA ratio in the diet on the expression of genes involved in CLA synthesis or affected by CLA in beef cattle. The complex pathways involving these genes are summarized in Figure 1.

Some of the genes studied are directly involved in fatty acid synthesis, such as acetyl-CoA carboxylase (*ACC*), fatty acid synthase (*FAS*), stearoyl-CoA desaturase (*SCD*), delta-6-desaturase (*Δ6D*), delta-5-desaturase (*Δ5D*) and fatty acid elongase 5 (*ELOVL5*). Other targeted genes are affected by CLA and can be related to lipid metabolism: activated protein kinase (*AMPKα*), adiponectin (*ADIPOQ*), sterol regulatory element-binding protein 1c (*SREBP-1c*), G protein-coupled receptor (*GPR43*), peroxisome proliferator-activated receptor γ and α (*PPARγ, PPARα*), statin (*STAT5*), adipose differentiation-related protein (*ADFP*), glucose transporter 4 (*GLUT4*).

In this context n-6 (sufflower oil) vs. n-3 (protected fish oil) PUFA effects on lipogenic gene expression were studied in bovine *longissimus dorsi* muscle of Aberdeen Angus and Belgian Blue breeds. These breeds were chosen due to contrasting attributes in terms of maturity (AA is an early maturing breed, while BB is late maturing breed)

2. Material and Methods

Animals, study design and tissue sampling

Spring-born early-maturing Aberdeen Angus × Friesian heifers (AAF, n=24) and late-maturing Belgian Blue × Friesian heifers (BBF, n=24) were reared from four months of age according to the heifer grass-based dairy calf to beef blueprint described by Keane and Drennan (22). Within breed, the animals were blocked on bodyweight and within block assigned at random to the control system (CON) or to continued supplementation with a safflower oil-containing supplement (SAFF) or protected fishoil-containing supplement (PFO). Thus, CON animals grazed a perennial ryegrass-dominant sward from July 1, and were offered a daily allowance of grass dry matter (DM) sufficient to ensure a consumption of 2.5% bodyweight (assuming 75% utilisation) for the whole group. They were housed November 30 and offered unwilted grass silage ad libitum and 1.0 kg standard concentrates per animal daily until turnout to pasture on April 1. Thereafter until slaughter at the end of October, they again grazed a perennial ryegrass-dominant sward. Housing and turnout dates were the same for SAFF and PFO animals as for CON animals. However, in each case, the animals received a supplement formulated to supply 50g lipid / kg total dietary DM (Table 1).

At pasture, the SAFF animals received a supplement allowance of 2.5 kg/425 kg bodyweight, while the PFO animals received an allowance of 1.82 kg/425 kg bodyweight. For both SAFF and PFO groups, the animals received a restricted daily grass allowance to ensure similar carcass growth as the CON group. Upon housing, SAFF animals received a restricted amount of wilted silage + 2.5 kg supplement/425 kg bodyweight while PFO animals received a restricted amount of unwilted silage + 1.82 kg supplement/425 kg bodyweight. These ration allowances were adjusted to ensure similar mean liveweight growth as the CON animals. Upon turnout, animals in the SAFF and PFO groups were managed as described for the previous summer.
At 21 months of age, animals were slaughtered and *longissimus dorsi* (LD) muscle was collected as decribed by Keady et al. (11)

RNA Extraction, cDNA Synthesis, and Real-Time Quantitative PCR

Total RNA was isolated from frozen muscle tissue using TRI reagent (Sigma-Aldrich Ireland Ltd., Dublin, Ireland) and immediately precipitated using isopropanol. The samples were then purified using the Zymo RNA purification Kit (Zymo Reasearch Corporation. The quantity and purity of RNA were measured using absorbance at 260 nm on a spectrophotometer (NanoDrop Technologies, Wilmington, DE). All RNA samples had a 260:280 nm absorbance ratio between 2,04 and 2,14.
The Agilent Bioanalyser with the RNA 6000 Nano LabChip kit (Agilent Technologies Ireland Ltd., Dublin, Ireland) was used to assess the 28S:18S ratio and the RNA integrity number (RIN) by automated capillary gel electrophoresis. One microgram of total RNA was reverse transcribed to cDNA, with random hexamers, using the High Capacity cDNA Reverse Transcription kit (Applied Bio- systems, Warrington, UK), according to instructions supplied, and stored at −80°C.
Real-time quantitative PCR (**RT-qPCR**) was used to measure gene expression. Primers were designed to amplify templates of a specific range between 70 and 200 nucleotides overlapping exon-exon junctions where possible, using the primer3 web-based software program (http://frodo.wi.mit.edu/primer3), while the primer specificity was checked using the BLAST search tool (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences of primers used for each gene were commercially synthesized (Sigma-Aldrich Ireland Ltd.).
In Table 1 the designed primer specifications for each gene are reported.

The stability of expression of candidate reference genes was investigated across all samples in the study. Reference genes included β-actin (*ACTB*), ribosomal protein S18 (*RPS18*), glyceraldeyde-3-phosphate dehydrogenase (*GAPDH*), ubiquitin conjugate protein (*UBIQ*) and B_2 microglobulin (*B2M*). The resulting expression data were analyzed using geNorm software (version 3.5, Excel add-in, Microsoft, Redmond, WA) as described by Vandesompele et al. (23) to test the overall stability of the chosen reference genes. The expression stability value was estimated (***M***) for each reference gene (the lowest *M* value being the most stable) using this program. The reference genes with the highest *M* values were excluded and geNorm determined the minimal number of reference genes required for calculating an accurate normalization factor. In the current study, the highest stability was obtained including 4 reference genes, *ACTB*, *RPS18*, *GAPDH* and *UBIQ* achieving a M value < 1.5.

All RT-qPCR reactions were performed using SYBR Fast Green mastermix (Applied Biosystems). Assays were carried out under identical conditions, and all samples were measured in triplicate using the Applied Biosystems Fast 7500 v2.0.1 instrument with the following cycle parameters (95°C for 15 s, 60°C for 60 s, 95°C for 15 s, and 60°C for 15 s). Primer and cDNA concentrations were optimized for each gene. The efficiency of the reaction was calculated using a 5-fold dilution series of cDNA to generate a standard curve. All PCR efficiency coefficients ranged between 0.8 and 1.20, therefore acceptable. The software package GenEx 5.2.1.3 (MultiD Analyses AB, Gothenburg, Sweden) was used for efficiency correction of the raw cycle threshold values, interplate calibration based on a calibrator sample included on all plates, averaging of replicates, normalization to the reference gene, and the calculation of quantities relative to the highest cycle threshold value.

Statistical analysis

Data collected from the experimental trial were checked for normality using the UNIVARIATE procedure of SAS (SAS Inst. Inc., Cary, NC). Where necessary, data were transformed using the TransReg procedure, by raising to the power of λ. Data were then subjected to Analysis of Variance for a split-plot design with block and breed (B) in the main plot and ration (R) and all interactions in the split-plot.

3. Results

The expression of 15 candidate genes was analysed in this study. These genes are involved in the pathways summarized in Figure 1.The genes can be divided into those encoding: 1) lipogenic enzymes such as *ACC*, *FAS*, *SCD*, *Δ6D*, *Δ5D* and *ELOVL5*; 2) factors involved in lipid metabolism such as *AMPKα*, *ADIPOQ* and *GPR43*; 3) transcription factors including *SREBP1c*, *PPAR γ* and *α* and *STAT5*; 4) lipid storage-associated proteins such as *ADFP*; and 5) energy metabolism such as *GLUT4*.
Table 3 summarises the effects of breed and dietary n-6 and n-3 PUFA ratio on the expression of candidate genes.

Effect of breed on gene expression

The expression of the gene encoding *ACC* was higher (p=0.013) in BB than in AA. In addition, *FAS* gene expression was higher (p=0.007) in AA than in BB.
(p=0.055) for SCD expression tended to be higher for BB than in the AA breed.
No effect of breed was found in the expression levels of the other genes examined.

Effect of oil supplementation

The expression of the gene encoding for *FAS* was increased (p=0.006) by supplementation with n-3 PUFA compared with both the control and the SAFF supplementation group. *SCD* gene expression showed a tendency (p=0.074) for the same effect, although this increase was not statistically significant.
Regarding the other genes encoding for lipogenic enzymes, an absence of significant relationships among n-3:n-6 PUFA ratio and *Δ5D*, *Δ6D* gene expression was showed, while a trend could be found between the n-3:n-6 PUFA ratio and *ELOVL5* (*P*= 0.198) and *GPR43* (*P*=0.199): gene expression. CLA supplementation resulted in both genes having higher expression among the n-3:n-6 ratios, with lower expression of these genes observed due to n-3 supplementation.
No effect of n-3:n-6 ratio was observed on the expression of the other genes analysed.

Breed X Oil supplementation

An interaction between breed and dietary n-3:n-6 ratio was observed for the *PPARα* gene: ω3 supplementation significantly reduced *PPARα* expression in AA, while it was increased in BB (*P*= 0.008).

SAFF supplementation increased the expression of *ADIPOQ* in AA but decreased it in BB (*P*=0.055).

4. Discussion

The effect of breed and dietary fatty acid intervention on shifts in the expression of genes related to CLA and lipid metabolism was examined in this study. The gene studied are categorised as follows: 1) lipogenic enzymes such as *ACC*, *FAS*, *SCD*, *Δ6D*, *Δ5D* and *ELOVL5*; 2) factors involved in lipid metabolism such as *AMPKα*, *ADIPOQ* and *GPR43*; 3) transcription factors including *SREBP1c*, *PPARγ* and *α* and *STAT5*; 4) lipid storage-associated proteins such as *ADFP*; and those involved in 5) glucose metabolism such as *GLUT4*.

Effect of breed on gene expression

ACC and *SCD* gene expression were higher in BB, in contrast with the expectation that early maturing breeds should show higher gene expression than late maturing cattle. In fact AA was expected to have higher levels of gene expression than BB, which is late maturing. Moreover the expression of genes involved in fatty acid synthesis was expected to be higher in AA also due to the higher fat content associated with this breed. For example, Barton et al., (24) reported the highest levels of expression of *ACC*, *FAS* and *SCD1* in intramuscular fat of AA and Holstein bulls when compared with other breeds and justified the results due to these breeds having increased fat depots.

It could be hypothesized that our results in the AA breed component of the crossbred animals used in the study. Moreover the literature reports that high intramuscular fat in Angus is positively related to higher *ACC* and *FAS* genes expression (24). Thus the quantity of fat might be another factor explaining the lower gene expression in AA crossbreed than BB crossbreed in our study.

In addition, Smith et al (25) reported that acetate incorporation into fatty acids in adipose tissue of Angus × Hereford and Red Poll steers increased between 10 and 16 mo of age and decreased with additional time on hay feed, and the same was found for *ACC* and *FAS* lipogenic enzyme activities. Thus the age of our animals (>16 months) and the long-fed could suggest that the sampling was done during the stage of decreasing *SCD* gene expression and therefore reduced lipogenesis for an early maturing breed such as AA compared to BB.

Also Chung et al. (26) showed a depressed rate in lipogenesis in long-term fed steers and concluded that the increase in *SCD* gene expression probably resulted in a proportional increase in lipogenesis only in the first period of feeding.

Moreover BB is characterized by muscular hypertrophy, which occurs because of mutations in the myostatin coding sequence as reported by McPherron, (27). The consequence of this, as explained by Keady et al., (11) is the higher glycogen metabolism in the muscle of BB breed. Thus, in a muscle where Acetyl-CoA is basic, if it is supposed to be a limiting substrate, the muscle could show higher *ACC* gene expression in the BB breed.

In contrast, *FAS* expression was higher in the AA breed. That may be due to the early maturity of this breed as reported above, to its pattern of fat storage. Moreover this result could also be related to the available concentration of Malonyl-CoA, which is a substrate for the FAS enzyme: It could be hypothesized that in an early maturing breed, the concentration of this substrate were higher than in the late maturing breed BB.

Effect of oil supplementation

Regarding the genes in group 1), an increase in *FAS* gene expression and an increasing trend in *SCD* due to n-3 PUFA supplementation were observed. No differences due to dietary treatment were found in *ACC, Δ5D*, *Δ6D* expression.

Our findings agree with work of Buchanan et al. (28) who reported an up-regulation of *FAS* gene expression in forage-finished heifers, and with those of Hiller et al. (19,29).

Regarding *SCD* gene expression, our findings were in contrast with Hiller et al. (19,29) and Waters et al., (15), who reported a significant decrease in *SCD* gene expression upon dietary n-3 fatty acid intervention. Also the work of Conte et al. (30) showed a decrease in *SCD* mRNA abundance after the replacement of sunflower oil (n-6 PUFA)

with linseed oil (n-3 PUFA) in lambs. Consistent with these findings, Buchanan et al (28) observed an up-regulation of *SCD* gene in animals fed with concentrates and a down-regulation after forage feeding.

However, gene expression can vary due to FA intervention in the diet, depending on tissue, breed and sex (29). The works of Hiller et al. (29) and Waters et al. (15) were carried out, respectively, with Holstein and Charolais and Limousine bulls, while in our study Aberdeen Angus and Belgian Blue x Fresian heifers were used. Thus a variation in *SCD* gene expression could be related to the choice of breed and sex of the cattle used in the experiment. Moreover Keating et al. (31) found that the bovine *SCD* gene promoter was downregulated by oleic acid, which is in line with our findings. Overall, the results in the literature are contrasting: for example Sessler et al. (32) showed *in vitro* that mRNA expression for *SCD* was decreased in a dose- dependent manner by addition of n-6 PUFA, linoleic acid and arachidonic acid to a murine cell line and also linolenic acid inhibited *SCD* gene expression in mouse adipocyte cell line.

Consistent with these findings, the work of McGettrick et al. (33) reported that grazing cattle supplemented with FO had lesser relative quantities of *SCD* mRNA in muscle and adipose tissue. Dietary effects on *SCD* gene expression are also mediated by effects on *SREBP-1c* and *PPARs* transcription factors and by hormones such as insulin and leptin (34). Waters et al. (15) reported that the effect of n-3 PUFA on *SCD* mRNA levels in bovine could be mediated by reduced *SREBP-1c* gene expression, while Biddinger et al (35) reported a reduced *SCD* gene expression by leptin with an independent mechanism of *SREBP-1c*. Sterol regulatory element-binding proteins 1c (*SREBP-1c*) also positively regulates the expression of genes encoding lipogenic enzymes including *ACC* and *FAS* (1,2,36,37). *PPARγ* and *SREBP-1c* gene expression in relation to different supplementation is discussed below.

Results of *ACC* gene expression in our study contrast with those of Hiller et al. (19), where *ACC* expression was decreased due to n-3 supplementation. The absence of effects on the expression of the other genes in the lipogenesis pathway (*Δ5D*, *Δ6D)* was instead in line with the study of Buchanan et al (28). To our knowledge, this is the first study on *ELOVL5* gene expression in relation to different dietary supplementation. No difference was found, in accordance with the absence of differences in the

expression of the genes encoding *Δ5D* and *Δ6D*, involved in the FA synthesis pathway immediately before and after *ELOVL5*.

Regarding group 2) including the genes *PPARγ*, *PPARα*, *SREBP-1c* and *STAT5*, no differences were found among the treatments. *SREBPs* are transcription factors for cholesterol and fatty acid synthesis (36), activating genes required for the synthesis of triacylglycerols and phospholipids (38). *PPARγ* is a key regulator of adipogenesis, cellular differentiation, insulin sensitization, atherosclerosis, and cancer (39) and promotes lipogenesis and fat storage. *PPARα* promotes instead β-oxidation in conditions of negative energy balance, (40,41,42).

Our results were in contrast with our own previous work (15), and that of Herrmann et al (43) and Buchanan et al. (28). The first study reported a negative relationship between the expression of *SREBP-1c* and n-3 supplementation. The second study reported instead a reduction in *PPARγ* gene expression due to CLA supplementation. Buchanan et al. (28) reported an up-regulation in gene expression of *PPARγ* in finished-forage heifers. However, our results were in line with Hiller et al (29), who reported an absence of relationship between *SREBP-1c* and different dietary supplementation in muscle and with Waters et al. (15), who found that n-3 PUFA had no effect on *PPAR α* gene expression.

Thus the absence of effect on treatments on *SREBP-1c*, *PPARγ* and *PPARα* could be related to a lack in sufficient variation in the supplementation, or to the tissue chosen for the analysis as suggested in a previous study by Hiller et al. (19).

PPARγ can activate *GLUT4* gene expression as reported by Wu et al. (44). Herrmann et al. (43) reported a reduction in *PPARγ* expression accompanied by an induced *GLUT4* gene expression after n-3 supplementation. The authors suggested an isomer specific influence of CLA on glucose and lipid metabolism that is genotype dependent and poorly mediated by *PPARγ*. In our work, *GLUT4* gene expression was not different among the treatment groups. This can be explained with the hypothesis suggested above by Herrmann et al.(43) or with the absence of effects of n-3:n-6 PUFA ratio on *PPARα* and *AMPKα* gene expressions, that are two of the main factors affecting *GLUT4* in muscle (45). Moreover no difference in *GLUT4* gene expression may have occurred due to the diets being isoenergetic as previously reported by Duehlmeier et al (46) and Peyron-Caso et al. (47) as only a change in the glucose levels in diet or blood could be related to shifts in *GLUT4* gene expression.

Due to the roles of *STAT5* in inflammation and adipogenesis, and the effects of CLA as a mediator of inflammation and body fat mass, a possible relationship with CLA supplementation was investigated.

Indeed, *STAT5* is involved in signaling pathways for cell proliferation, apoptosis, cell differentiation and inflammations (48). It is strictly related to lipid metabolism: an activation of the *STAT5/PPARγ* pathway by GH affects adipogenesis (49).

However, *STAT5* gene expression did not show any difference among the treatments, suggesting its involvement in regulating pathways different than those of CLA.

Regarding group 2, *AMPKα*, *ADIPOQ* and *GPR43,* no relationships were found among the expression of these genes and supplementation in the diet. The results were in agreement with the findings of Kelly et al (50), who reported an absence of supplementation effects on *AMPKα* gene expression. Moreover, *AMPKα* could be affected by *GPR43* and *ADIPOQ*, thus the absence of an effect on the last gene expression could explain the lack in differences in *AMPKα*. Our findings were however in contrast with those of Buchanan et al (28) and Hiller et al (29). Buchanan et al (28) reported that a concentrate-finishing diet was related to a decrease in *ADIPOQ* gene expression and Hiller et al. (29) showed that a n-3 FA intervention down-regulated *ADFP* gene expression. It could therefore be hypothesized that a lack of sufficient differences among the diet applied in this study could explain the lack of differences in the expression of genes analysed.

Breed X Oil supplementation

The differences found in *PPARα* gene expression between two breeds among the treatments were according to the breed skills: AA muscle are known to have greater levels of fat while BB has a strong muscular growth and lean meat (27). Indeed AA showed a reduction in *PPARα* expression, possibly related to β-oxidation and lipid catabolism, while the opposite occurred for BB. This result also supported the hypothesis that the sampling was carried out when a high lipogenesis occurred, so when *SCD* gene expression in AA animals has already decreased.

ADIPOQ results could be explained by the role of this gene as an enhancer of fatty acid β-oxidation and insulin response. Our findings showed that CLA in AA increased *ADIPOQ* gene expression: that could be related to features of the particular breed used (i.e., high marbling and intramuscular fat) and to the CLA effects against inflammations (*c9,t11*) and for the reduction of body fat mass (*t10,c12*) (53, 54).

5. Conclusions

To our knowledge, this is the first study screening a complete set of genes involved in CLA metabolism: our study aimed to investigate the effect of breed and of n-3/n-6 FA supplementation on the expression of 15 genes. The Aberdeen Angus and Belgian Blue breeds were chosen due to their different impetus in maturing and storing fat. The breed effect was showed in *ACC* and *SCD* gene expression, that were higher in BB (P=0,013; P= 0,055), and in *FAS* expression, that was higher in AA (P=0,007).
The diet effect on gene expression was found after n-3 FA supplementation that showed a positive correlation with *FAS* and *SCD* expressions (P=0,006; P=0,074).
Finally an interaction between breed and dietary n-3:n-6 ratio was reported for *PPARα* (P= 0,006) and *ADIPOQ* (P=0,055): n-3 FA reduced *PPARα* expression in AA, while it increased the same gene expression in BB. Moreover *ADIPOQ* gene expression was positively correlated in AA and lower in BB with CLA.
Thus this study highlighted the significance of breed and of alimentary n-3 and n-6 FA intervention on the muscle expression of genes related with CLA metabolism.
However more studies are needed to investigate the underlying mechanisms and the effects on the genes not directly involved in CLA synthesis.

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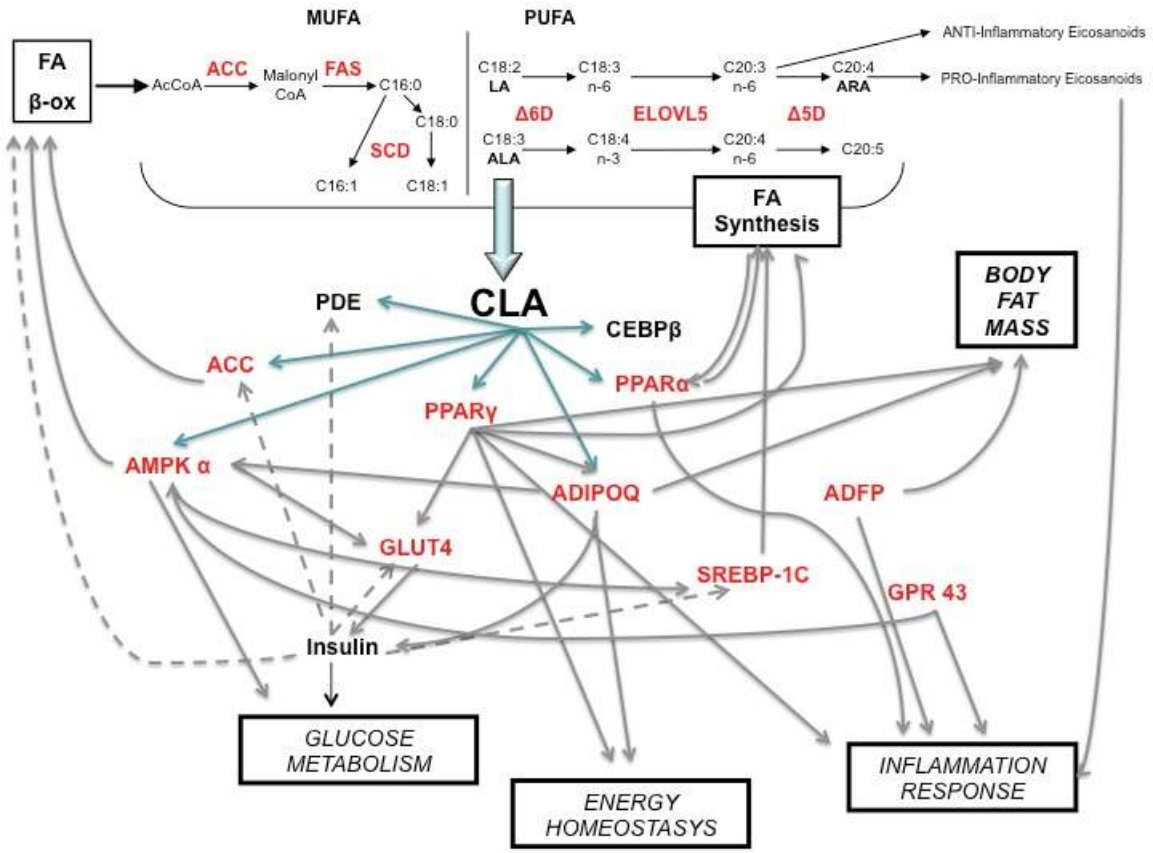

Figure 1. Genes involved in the CLA pathways.

Table 1. Composition of Experimental Rations

Diet		kg/tonne
Control concentrate (Winter)[1]		
	Barley	865
	Soya	65
	Molasses	45
	Min/Vit-1	25[a]
CLA Concentrate[2]	Safflower Oil	200
	Molasses	50
	Min/Vit	25[b]
	Maize meal	725
Omega 3-PUFA Concentrate[3]	Farm First	915
	Molasses	51
	Min/Vit	34

[1]CON; [2]CLA; [3]ω3; a= Standard Grange; b=80,000 IU vitamin E/kg

Table 2. Bovine oligonucleotide primers used for qPCR (Reference and Target genes)

Gene	Primer sequence	PCR efficiency	Size (bp)	Accession No.
ACTβ	Forward: GACACCGCAACCAGTTCGCCAT	0.91	194	NM_173979.3
	Reverse: AGCCTCATCCCCCACGTACGA			
UBIQ	Forward: AGATCCAGGATAAGGAAGGCA	1.06	98	NM_174133
	Reverse: GCTCCACCTCCAGGGTGAT			
RPS18	Forward: ACCAACATCGATGGGCGGCG	1.11	150	NM_001033614.1
	Reverse: CACACGTTCCACCTCATCCTCGG			
GAPDH	Forward: GATTGTCAGCAATGCCTCCT	0.80	144	NM_001034034
	Reverse: CCATCCACAGTCTTCTGGGT			
ACC	Forward: GAGCTGAACCAGCACTCCCGA	0.90	215	NM_174224.2
	Reverse: TGCAAGCCAGACATGCTGGATCTCA			
Δ5D	Forward: AGTTCAGGCCCAGGCTGGCT	0.99	164	XM_612398.5
	Reverse: TGGGCTTGGCATGGTGCTGG			
Δ6D	Forward: TGCCAACTGGTGGAACCATCGC	0.83	189	NM_001083444.1
	Reverse: GGCGGCCCGATCAGGAAGAAGTAC			
FAS	Forward: GCCAGCGGGAAGCGTGTGAT	1.19	235	NM_001012669.1
	Reverse: CGATGGCAGCCTGGCCTACG			
SCD	Forward: CTACAAAGCTCGGCTGCCTCTGC	0.83	202	NM_173959.4
	Reverse: TTTGACAGCTGGGTGTTTGCGC			
SREBP1c	Forward: TGGGCACCGAGGCCAAGTTGAAT	0.93	170	NM_001113302
	Reverse: TCCACTGCCACAAGCCGACA			
PPAR γ	Forward: AGGATGGGGTCCTCATATCC	0.90	121	NM_181024
	Reverse: GCGTTGAACTTCACAGCAAA			
AMPKα	Forward: GTCAAAGTCGGCCAAATGAT	0.80	104	BC_153842
	Reverse: CCTCCGAACACGCAAATAAT			
ADFP	Forward: GTCTGTCCTGGCTGGAGTGGAAGAG	0.94	150	NM: BT029909.1
	Reverse: TGTTGGACAGGAGGGTGTGGCA			
PPARα	Forward: GGTAACCGGAAGGCTACTCC	0.93	111	NM_001034036
	Reverse: CCTCATTTCCCAAGTCCTGA			
GPR43	Forward: GACGCAGATGCAAAGAAACA	1.07	107	NM: FJ_562212
	Reverse: CCAGGAACATCCCTAGTCCA			
GLUT4	Forward: ACCTTATGGCCACTCCTCCT	0.86	180	NM_174604
	Reverse: CTCAGCCAACACCTCAGACA			
ADIPOQ	Forward: CCAAGCAGCACAAAGTGAAA	0.79		NM_174742.2
	Reverse: AGACTGTCCTGGGAACATGG			
ELOVL5	Forward: CGCCACACTTAACAGCTTCA	0.98	174	NM_001046597.1
	Reverse: AAGGTACACGGCCAGATGAC			
STAT5 B	Forward: TGCATCCGCCATATTCTGTA	0.88	137	NM_174617.3
	Reverse: AGTCGCAGCTCCTCAAATGT			

Table 3. Effects of breed and ratio on the gene expression in *longissimus dorsi* muscle of heifers.[1]

	AAF			BBF			*P*-value		
Gene	NONE	CLA	ω3	NONE	CLA	ω3	B	R	BxR
ACC	1.752	1.657	1.938	2.319	2.130	1.851	0.013	ns	ns
FAS	0.810	0.945	0.973	0.728	0.835	0.900	0.007	0.006	ns
SCD	2.08	1.72	2.19	2.39	2.01	2.98	0.055	0.074	ns
Δ6D	1.241	1.134	1.265	1.226	1.228	1.210	ns	ns	ns
Δ5D	2.031	1.763	2.126	2.247	1.991	1.952	ns	ns	ns
EVLOVL5	0.731	0.963	0.867	0.913	0.973	0.789	ns	ns	ns
ADIPOQ	0.903	1.075	1.015	1.075	0.622	0.978	ns	ns	0.055
GPR43	-0.484	-0.603	-0.272	-0.455	-0.572	-0.443	ns	ns	ns
AMPKα	0.972	0.909	0.927	0.922	0.866	0.907	ns	ns	ns
SREBP1c	0.926	1.311	0.903	0.972	0.961	1.091	ns	ns	ns
PPARγ	1.114	1.107	1.078	1.130	1.179	1.085	ns	ns	ns
PPARα	1.097	1.002	0.959	0.994	1.024	1.165	ns	ns	0.008
STAT5	-0.170	-0.114	-0.056	-0.151	-0.204	-0.236	ns	ns	ns
ADFP	1.177	1.206	1.238	1.221	1.332	1.152	ns	ns	ns
GLUT4	0.869	0.997	1.090	0.992	0.991	1.054	ns	ns	ns

[1]Gene expression values were normalized to the reference gene after adjustment for efficiencies and interplate variation and converted to values

relative to the greatest cycle threshold (Ct) within each data set.
[2]ns = not significant; NONE = control diet; CLA = diet supplemented with CLA; ω3 = diet supplemented with ω3; B = breed; R = ratio
[3]*AA* = Aberdeen Angus; *BB* = Belgian Blue.

XIV. GENERAL CONCLUSIONS

Due to the healthy properties exerted by the *c9,t11* and *t10,c12 CLA*, these isomers could be considered healthy factors available in dairy products and meat from ruminants.

Dairy foods are essential for body metabolism and some components are involved in healthy processes: the synergy among CLA, vitamin D and calcium could be effective against cancer, in the prevention of atherosclerosis, in body fat mass reduction and insulin resistance control.

From our study on CLA content in Italian foods, a higher content in cheese than in meat was reported. Therefore the consumption of milk and its derivatives should be encouraged to improve daily CLA intake in humans, taking care of the implications in saturated saturated fatty acid intake, and the feeding straegies should be improved to enhance the CLA content in meat.

Comparing the *c9,t11* and *t10,c12* isomer contents found in our work with data from other countries, some differences were found, due to the different feeding strategies and food processing methods.

That confirmed that CLA food data produced on a National scale were necessary to implement the knowledge concerning the CLA intake needed for epidemiological studies.

As a matter of fact our studies showed that the CLA intake in Italy could not reach the proposed needed amount of 0.8-3 g/day reported by Parish et al. (2003) to exert the health benefits in human.

Thus more strategies are needed to improve the CLA amount in foods, particularly with genetic approaches.

A large number of genetic studies showed the undergoing mechanisms involved in CLA synthesis. In our research some genes involved in CLA synthesis were found to be affected by CLA or ω3 supplementation within the diet, and the CLA synthesis in tissues was improved.

Thus a nutri-genomic approach is needed to identify the best and easiest strategies enhancing the amount of CLA isomers in foods.

More information on these issue could allow the farmers and the LRT to provide foods with higher nutritional values, thus improving consumers satisfaction and public health.

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