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Effects of conjugated linoleic acids (CLA) and essential fatty acids (EFAs) on oxidative and inflammatory status in bovine mammary epithelial cells (BME-UV1)

S.S.D. AGR/18- Animal Nutrition and Feeding

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Abstract

During the transition period, dairy cows typically experience oxidative stress caused by drastic physiological changes. Oxidative stress was identified as an underlying factor of dysfunctional inflammatory responses. The use of CLA and EFAs has proved a valuable management tool in livestock field, however, it has not been well studied their antioxidant and anti-inflammatory properties and their effectiveness in preventing oxidative stress and inflammation in dairy cows. The purpose of this research was to investigate and to better understand how the biologically active CLA isomers [cis-9,trans-11 and trans-10,cis-12], and EFAs [(α)-linolenic acid, (γ)-linolenic acid and linoleic acid] can affect the oxidative and inflammatory cellular state and so clarify their mechanism of action in BME-UV1. Three independent *in vitro* studies were conducted. The first study evaluated the antioxidant effect of CLA isomers, the second concerned the comparison of the antioxidant effect between CLA and other EFAs, the last compared the anti-inflammatory effects of CLA and EFAs. The results showed antioxidant and anti-inflammatory proprieties of CLA and EFAs. All fatty acids were responsible in the reduction of lipid peroxidation and down-regulated gene expression of pro-inflammatory cytokines. These fatty acids could reduce the incidence of oxidative stress and inflammatory process typical of transition period.

1. Introduction

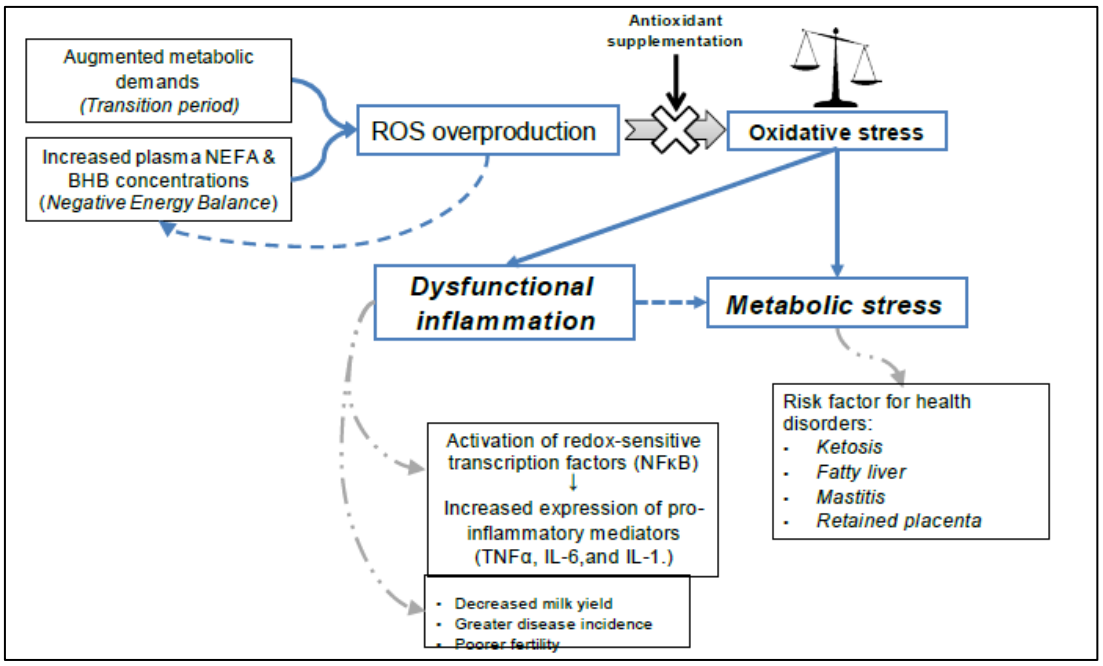
1.1 Oxidative Stress and Inflammation in Dairy Cow

The possibility that dairy cows may experience oxidative stress has been widely documented (Bernabucci et al., 2002, 2005; Castillo et al., 2006). As for humans also for dairy cow oxidative stress has been considered one of the factors associated with metabolic and infectious diseases that frequently occur in the transition period (Miller et al., 1993). In fact, is exactly during the transition period that animals experience oxidative stress. The transition period is comprised between three weeks before and three weeks after calving (Grummer, 1993; Drackley, 1999). The animals during this period have difficulty to meet the high energy demands of end pregnancy and early lactation, and often fail to take with the food the energy needed and for this reason must use their energy reserves. When this condition occurs, the animals undergo a negative energy balance (**NEB**). To compensate the energy shortage, the lipomobilization of fatty acids from tissues then starts (Figure 1). This increases the concentration of non-esterified fatty acids (**NEFA**) in the blood. Circulating NEFA are up-taken by the liver, and then converted into energy via oxidation (Sordillo and Raphael, 2013). However, when the concentration of NEFA exceeds the conversion capacity of hepatocytes, the liver function is impaired and increase the levels of ketone bodies and β-hydroxybutyrate (**BHB**) in the blood. In this way, the exhausting NEFA metabolism invalidates liver function but also fosters and increases the reactive oxygen species (**ROS**) production (Schönfeld and Wojtczak, 2008). So, the increased concentration of ROS in combination with a reduced concentration of antioxidants that occurs precisely in this physiological stage (Benabucci et al., 2005; Sordillo et al. 2009) expose the organism to the condition of oxidative stress. Moreover, the oxidative stress phenomenon favors an extra lipolysis, helping to increase the concentration of NEFA in the circulation, thus creating a vicious circle (Abuelo et al., 2015).

In dairy cattle, the inflammatory phenomenon is a state that occurs particularly during the transition period and is associated to a body's reaction to infectious problems, parasites and metabolic diseases; the release of pro-inflammatory cytokines would seem the factor common to these causes (Grimble, 1990). The inflammation usually arises as a resolutely response to an insult, but when it lasts for a long time can have a negative effect on well-being and on the animal's profitability. In fact, inflammation is associated to the reduction of dry matter intake, the impairment of liver function, the reduction of milk yield and fertility of the

dairy cow (Bertoni et al., 2015). Inflammation in periparturient cows could develop without clinical signs and can be associated, in particular, to animals with a severe NEB, with a lower body condition score (**BCS**), and with greater level of BHB in the blood (Bertoni et al., 2008). The inflammatory condition was associated to reduced reproductive performances, and to reduced energy efficiency (Trevisi et al., 2010). The increased concentration of NEFA in the blood that occurs in this period, are one of the factors that predisposes the animals to the inflammatory condition (Contreras and Sordillo, 2011).

Figure 1. Development of oxidative stress and inflammation in dairy cow



From Abuelo et al. (2015).

Studies on humans have claimed that the high concentration of NEFA leads to a chronic inflammatory condition, in particular obese subjects had greater concentrations of tumor necrosis factor-alpha (**TNF-α**) and interleukin-6 (**IL-6**) (Johnson et al., 2012). The great concentration of NEFA modifies the blood lipid pool in favor of saturated fatty acids, such as palmitate and stearate, and monounsaturated acids, oleic acid in particular (Sordillo and Raphael, 2013). These fatty acids activate toll-like receptor-4 (**TLR-4**) receptor in immune cells and not, favoring the consequential activation of nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-Kβ**) which translocates from the cytoplasm to the nucleus. When the NF-Kβ reaches the nucleus, it increases the expression of TNF-α, Interleukin-1 (**IL-1**) and IL-6 pro-inflammatory cytokines. These fatty acids, particularly

palmitic acid, have an antagonistic role as activators of TLR-4 receptors. In fact, normally, the TLR-4 receptor is stimulated by the lauric acid which is the main component of the lipid A associated with bacterial lipopolysaccharide (**LPS**) an inducer of inflammatory process (Sordillo et al., 2009).

Studies carried out until today have shown that oxidative stress and inflammation may be mitigated through nutritional strategies. In particular, among the substances most commonly used there are vitamin E and selenium, the use of which has allowed to help the oxidative system of cells and strengthen the immune system in the peripartum period. In the last period the scientific attention has been oriented towards the use of fatty acids such as conjugated linoleic acid (**CLA**) and essential fatty acids (**EFAs**). In fact, many human studies have shown beneficial properties of this substance in attenuating inflammatory processes, in particular through the use of omega-3 and CLA. In the livestock sector, the use of these fatty acids has helped to improve the productive and reproductive performance of dairy cow. For example, the CLA reduce synthesis of milk fat and have proven to be suitable in inducing energy savings in lactating animals (Baumgard, 2005). Moreover, the EFAs have helped the improvement of reproductive performance of cows (Leroy et al., 2014; Silvestre et al., 2011).

1.2 Oxidative Stress

1.2.1 ROS formation and oxidative stress

With the generic term of free radicals is denoted any molecular species capable of independent existence and to interact with other macromolecules by donating or accepting electrons (Lobo et al., 2010). Free radicals are intermediate products of the various biochemical reactions of the organism; in particular, radicals containing oxygen, identified as ROS, are formed from cellular respiration reactions. The oxidative reactions are part of the normal cellular aerobic metabolic processes, in whom the oxygen is the final acceptor in the electron's transport chain which leads to the formation of ATP in the mitochondria. Precisely, during the flow of electrons, some of these can react directly with the oxygen originating the ROS (Giovannini et al., 2006). The formation of ROS can occur also in response to the biotransformation by the cytochrome P-450 of foreign compounds, potentially harmful for the organism such as toxins, drugs, environmental factors like high concentrations of salts or

ultraviolet radiation. Also inflammatory factors can stimulate ROS production. In this case, it was observed that some cells of the immune system, such as macrophages, neutrophils and other specialized cells, generate radicals like superoxide anion ($^{\cdot}O_2^-$) and H_2O_2 by the enzyme system of the NADPH oxidase (Giovannini et al., 2006).

The main ROS produced during the respiration process is $^{\cdot}O_2^-$. The $^{\cdot}O_2^-$ is formed when molecular oxygen receives one electron:

$$\mathbf{O_2 + e^- \rightarrow {}^{\cdot}O_2^-}$$

Superoxide anion has a strong capacity of interaction. It may interact with other molecules generating other types of ROS. Generally, $^{\cdot}O_2^-$ is catalyzed by superoxide dismutase (**SOD**), which makes it less dangerous. From this reaction oxygen and H_2O_2 are released:

$$2^{\bullet}O_2^- + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

However, in other cases ROS can interact with certain transition elements like iron (Fe^{3+}) and copper (Cu^{2+}). In this case hydroxyl radicals ($OH^{\cdot}$) are formed:

$$^{\bullet}O_2^- + H_2O_2 \xrightarrow{Fe^{3+} \text{ or } Cu^{2+}} O_2 + OH^- + OH^{\bullet}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

Also under normal physiological conditions the free catalytic iron can significantly contribute to the production of ROS being it sequestered by various metal-binding proteins (Kakhlon and Cabantchik, 2002).

ROS can have the function of signaling molecules in biological processes thus contributing to the adaptation of living organisms to stress conditions such as environmental and oxidative changes (Schieber and Chandel, 2014).

For example, H_2O_2 is implicated in the activation of multiple sensors and pathways responsible for regulation of transcription factors, among which genes involved in the repair and maintain cellular homeostasis, for the control of cellular functions (such as cell proliferation, differentiation and apoptosis), and for the detoxification of oxidizing molecules (Espinoza-Diez et al., 2015).

Cells have also developed a sophisticated antioxidant system able to limit the amount of oxidizing agents to prevent damages that may result, and capable to maintain a correct organism redox balance. Normally, the correct balance between oxidants and antioxidants is in favor of antioxidants, however, when for environmental reasons or also following deficiencies of antioxidants in the diet the antioxidants/oxidants balance is broken, takes over an oxidative imbalance defined as a pathological condition and known as *oxidative stress* (Betteridge, 2000). The status of oxidative stress was associated with many pathological conditions in humans (Lobo et al., 2010).

Diseases related to oxidative stress are due to the excessive reactivity of ROS with the main cellular targets, such as lipids, proteins, DNA and RNA. Resultant effects are related to altered cellular activity due to the inactivation of enzymes and receptors, changes in the lipid peroxidation or in the protein synthesis and even genetic mutations (Costa et al., 2007).

1.2.2 Lipid peroxidation

The lipoperoxidation phenomenon is one of the effects of related unchecked oxidative stress. Hydroxyl radical and hydroperoxyl are the two most predominant ROS that can react particularly with cellular lipids. The lipid attack of ROS causes the abduction of hydrogen from a carbon of lipids and the insertion of oxygen. Specifically, the lipids that contain a double carbon-carbon bond are more susceptible (Ayala et al., 2014). From lipid peroxidation derived different products, among these the main are lipid hydroperoxides (**LOOH**) and several aldehydes: 4-hydroxynonenal, propanal, hexanal and malondialdehyde (**MDA**). The radicals which are derived from the lipid peroxidation, as well as MDA, are harmful because can cause protein oxidation or reacts with DNA, forming other segments with mutagenic potential (Dalle-Donne et al., 2006; Marnett et al., 1999).

The MDA quantification is considered a useful marker for lipid peroxidation, specifically for omega-3 and omega-6 fatty acids (Ayala et al., 2014). In fact, MDA is one of the most prevalent and trustworthy markers that indicates the level of oxidative stress.

1.2.3 Protein Oxidation

The protein segments more susceptible to damage are multiple side-chain and backbone sites (Davies, 2016). Modifications that may result from the attack of ROS to protein affect cleavage of protein backbone, generation of carbonyl derivatives and formation of cross-linked protein complexes (Berlett and Stadtman, 1997; Tretyakova et al., 2015). The targets are not only the proteins or polypeptides, but also free amino acids, although usually are present at lower concentrations (Davies, 2016).

A good way to evaluate the oxidation of proteins is the quantification of protein carbonyls (Ahmad et al., 2008). These appears through side-chain oxidation of proline, arginine, and lysine. The presence of reactive aldehyde or ketone groups in the protein makes it easy to quantify because these groups react with hydrazine to form hydrazone, and are considered a reliable markers of oxidative stress (Barelli et al., 2008).

1.2.4 Oxidative damage to DNA and RNA

Both DNA and RNA are targets of oxidative stress, in particular the mitochondrial DNA (Lobo et al., 2010). DNA damage, occurring as a result of oxidant attack, includes single-strand breaks, double-strand breaks, misalignments, chemical modifications of the bases or sugars and inter- or intra-strand cross-links (Maynard et al., 2009). Many DNA damages are related to the oxidation of the bases. The base with the lower oxidative potential is guanine and this condition makes this base particularly vulnerable to oxidation (Neeley and Essigmann, 2006).

From this oxidative process derives various products of which the most famous and studied is the 8-hydroxy-2-deoxyguanosine, used as a biological marker of oxidative stress (David et al., 2007).

1.2.5 Antioxidant system

Cellular antioxidant system includes both enzymatic and non-enzymatic molecules (Birben et al., 2012). SOD, catalase (**CAT**), thioredoxin reductase (**TrxR**), and peroxiredoxin (**Prx**) represent the first line of cellular defense. These enzymes have the task to limit the damage of membrane lipids and biological macromolecules by ROS (Celi, 2010; Giovannini et al., 2006). In particular, SOD, as seen previously, catalyzes the dismutation of superoxide to hydrogen peroxide and this is considered absolutely the first workhorse against oxidizing (Halliwell and Chirico, 1993). The CAT catalyzes the conversion of hydrogen peroxide to water and molecular oxygen (Schreibelt, 2007). TrxR performs its main antioxidant activity providing the electrons at thiol-dependent Prx to remove reactive oxygen (Pannala and Dash, 2015).

Nevertheless, these enzymes in the execution of their protective action, do not give a perfect hedge against possible risks. For this reason, it is necessary a second line of defense represented by the enzymes glutathione peroxidase **(GPx1)**, glutathione reductase (**GR**), glutathione S-transferase (**GST)**, aldo-keto reductase (**AKRs**) and aldehyde dehydrogenases (**ALDHs**) (Giovannini, et al., 2006). GPx1 is a ubiquitous enzyme present at the cellular level in the mitochondria and cytosol, while his plasma level is considered an indicator of oxidative status (Bernabucci et al., 2002; Tüzün et al., 2002). It contributes to the defense by catalyzing the reduction of hydrogen and lipoperoxides (Halliwell and Chirico, 1993). The antioxidant role of GST concerns in the detoxification of electrophilic reactive compounds, including environmental toxins or products of oxidative stress, through the conjugation with glutathione (Espinoza-Diez et al., 2015).

The metabolites formed by the reaction of detoxification are then removed from the cell to the extracellular environment by efflux pumps, by the carrier glutathione S-conjugate (Giovannini et al., 2006). The AKRs are a unit of NAD(P)H-linked oxidoreductases, which reduces aldehydes and ketones to their respective primary and secondary alcohols (Penning, 2004). ALDHs mitigates oxidative/electrophilic stress by metabolizing endogenous and exogenous aldehydes (Singh et al., 2012).

The non-enzymatic antioxidants can be found in plasma and in the extracellular and intracellular fluids (Celi, 2010). The antioxidant power of the serum is linked to the presence of non-enzymatic endogenous antioxidants such as glutathione, protein-thiol groups together

with exogenous antioxidants (α-tocopherol or Vitamin E, uric acid and β-carotene; Halliwell and Gutturidge, 1993).

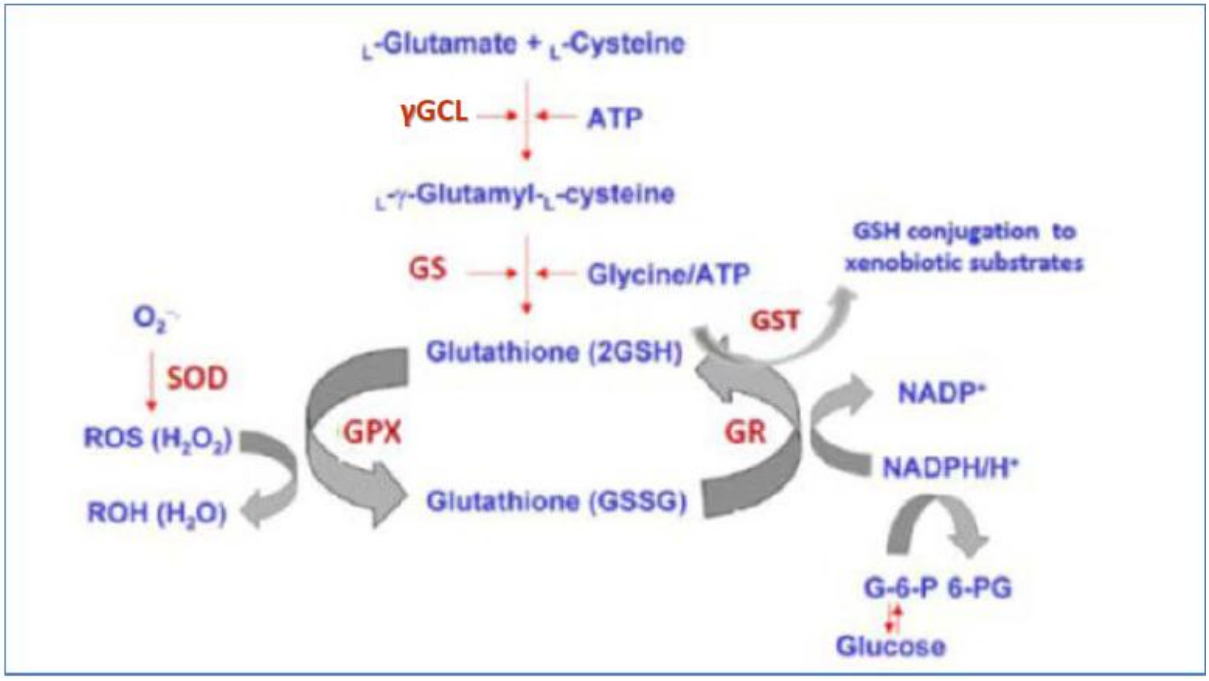
Glutathione is the major endogenous antioxidant and it plays a very important role in protecting cells against oxidative stress and toxic agents. This tripeptide consists of glutamic acid, cysteine and glycine and plays his antioxidant action in cellular defense lines, by acting as a substrate or co-substrate in enzymatic reactions and can also react directly, as scavenger, with free radicals or lipid peroxides (Giovannini et al., 2006; Lu, 2013). For example, glutathione can directly react with $\cdot O_2^-$ and some other ROS, but its indirect ROS-scavenging functions, such as revitalizing other antioxidants, is likely more important. It can reduce de-hydro ascorbic acid which is formed in the reconversion of α-tocopherol to tocopheroxyl radical, a lipophilic chain breaking antioxidant, which interacts with the poly-unsaturated-acyl groups of lipids, stabilizes membranes and scavenges various ROS and lipid oxy-radicals (Espinoza-Diez et al., 2015). Several researches conducted on different types of mammalian tissues, showed that GSH is ubiquitous in almost all cells at concentrations of 1 to 10 mM (Espinoza-Diez et al., 2015).

In cells glutathione is in two forms: reduced (**GSH**) and oxidized (**GSSG**) form (Figure 2). During normal cellular oxidative conditions predominates GSH, which is 98% of the total glutathione. Eukaryotic cells possess three large reserves of GSH. The increased amount of GSH is stored in the cytosol (about 80-85%), the 10-15% is located in the mitochondria and the remaining part is within the endoplasmic reticulum. As previously reported, the antioxidant function of GSH is utilized largely by GPx1-catalyzed reactions, which reduce hydrogen peroxide and lipid peroxide, and as a result of this use, GSH is oxidized. GSSG in turn is reduced back to GSH by the enzyme GR, at the expense of NADPH, forming a redox cycle. The form of GSH within the cell is linked to a specific ratio: [2GSH]/GSSG. However, when the GSSG concentration exceeds the GSH cellular concentration, the cell maintains the redox equilibrium actively removing the GSSG outside. Otherwise, GSSG may react with sulfhydryl proteins forming disulfide mixed. These conditions can be defined extreme, for example when the cell is under a condition of oxidative stress (Lu et al., 2013).

The levels of GSH inside the cell are not dependent only by his recycle, it can also be synthesized directly. The *de-novo* synthesis of GSH occurs in two ATP dependent steps: the formation of γ-glutamylcysteine from $_L$-glutamate and $_L$-cysteine and formation of GSH from γ-glutamylcysteine and glycine. The first step of GSH biosynthesis is the rate limiting and is

catalyzed by the enzyme γ-glutamylcysteine Ligase (**γGCL**) which is composed of 2 subunits: a. heavy or catalytic (about 73 kDa) and b. light or modifier (about 31 kDa). The second step of GSH synthesis is catalyzed by glutathione synthetase (**GS**, also known as GSH synthase). GS is composed of two identical subunits (about 118 kDa) and is not subjected to feedback inhibition by GSH (Espinoza-Diez et al., 2015; Lu et al., 2013).

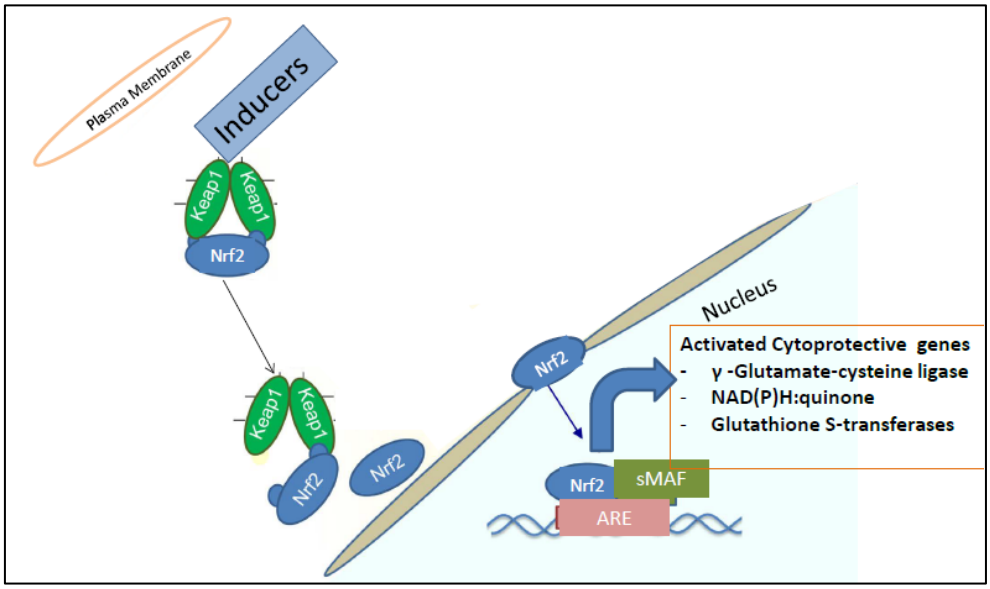
Figure 2. Activities of antioxidant enzymes with GSH training and recycling.



From Guilford and Hope (2014), modified

The gene expression of γGCL like other antioxidant enzymes such as GST and NAD(P)H:quinone oxidoreductase, are induced by the transcription factor nuclear factor-erythroid 2-related factor 2 (**Nrf2**). Nrf2 is the major regulator of cytoprotective responses to oxidative stress, the stimulation of its activity is a therapeutic target because it can reduce oxidative damage (Cuadrado et al., 2009). Normally, Nrf2 is sequestered in the cytosol by Kelch-like ECH-associated protein 1 (**Keap1**) homodimer (Figure 3), which facilitates its ubiquitination and proteasome degradation. Some inductors like some fatty acids (**FAs**), reacting with the cysteine residues present in Keap1 determines the Nrf2 release and its nuclear translocation. So, Nrf2 in the nucleus, heterodimerizes with small Maf (**sMAF**) proteins and binds to the antioxidant response element (**ARE**), by activating the expression of a battery of cytoprotective genes (Espinoza-Diez et al., 2015).

Figure 3. Antioxidant protective mechanism induced by Nrf2



From Espinoza-Diez et al. (2015), modified

1.3 Inflammation

1.3.1 Inflammatory process

Inflammation, from the Latin language *inflammatio*, is an innate immune response of the body with protective and reparative effects in response to various biological, chemical and physical insults (Mogensen et al., 2009). The classic signs of inflammation are the heat (*calor*) following the increase of temperature for hyperthermia and increase of cellular metabolism, pain (*dolor*) for biochemical changes, redness (*rubor*) as a result of increased vascularity, swelling (*tumor*) due to edema, and finally the loss of function (*functio laesa*) of the affected area (Ciaccia, 2011).

Two inflammatory types can be recognized: acute inflammation, which is the first response of the body to harmful stimuli and induces an increase in the flow of plasma and leukocytes to the damaged area; and chronic inflammation, which is a long-lasting inflammatory process, which can result from an unresolved acute inflammation, and it is characterized by the persistence of the triggering agents in the inflammatory processes and persistent tissue destruction and repair attempts (Murakami and Hirano, 2012).

There is another type of inflammation: the chronic systemic inflammation, for which there is a growing interest (Bertoni et al., 2015). This kind of inflammation is due to a continuous release of inflammatory mediators (pro-inflammatory cytokines) by immune cells and promotes chronic activation of the innate immune system (Newton and Dixit, 2012). In several human studies there is a general concept that chronic inflammation can be a major cause of the development of a wide variety of diseases, such as type-2 diabetes, cardiovascular diseases, cancer and neurodegenerative disease (Medzhitov, 2008; Rodríguez-Hernández et al., 2013).

The main actor of the inflammatory process is the innate immune system (Hato and Dagher, 2015). However, even the non-professional cells, such as epithelial cells, endothelial cells and fibroblasts can contribute.

The trigger of inflammation occurs when cells of the innate immune system identify some endogenous molecules released from stressed or dying cells termed damage-associated molecular patterns (**DAMPs**) or also pathogen-associated molecular patterns (**PAMPs**). DAMPs and PAMPs are intercepted through specific pattern-recognition receptors (**PRRs**) (Land, 2015).

The action of the inductors of PRRs, pushes the production and release of inflammatory mediators. Among these there rae: vasoactive amines (histamine and serotonin), responsible for immediate response and short duration of inflammation, including vasodilation, induce the increase of vascular permeability, and smooth muscle contraction (Qin et al., 2013); complement proteins (Markiewski and Lambris, 2007); eicosanoids, like thromboxanes, leukotrienes and prostaglandins (Ricciotti and FitzGerald, 2011), chemokines and pro-inflammatory cytokines (**PICs**) (Turner et al., 2014).

1.3.2 Pro-inflammatory cytokines

The inflammatory process involves the release of pro and anti-inflammatory cytokines, in particular, many studies have linked the close relationship between high presence of PICs and the increased pathogenicity of diseases (Tian et al., 2014). PICs include TNF-α, IL-1 and IL-6.

These signaling molecules are small proteins (ranging from 6 to 51 kDA) that can have a local or systemic action and can bind to specific receptors in an autocrine, paracrine or endocrine manner. The cytokines production and release can happen through the activation in

response of a stimulus (Arango Duque and Descoteaux, 2014; Jaffer et al., 2010). A classic stimulus that favors the production of PICs (Figure 4) is the cell stimulation with LPS of gram-negative bacteria. LPS contains the lipid-A which has highly inflammatory action on immune system cells. LPS can bind with cluster of differentiation 14 (**CD14**) or TLR-4 of tissue macrophages causing the detachment of NF-Kβ from inhibitor of kappa β (**IKβ**) units binders and its activation. Thus, the activated NF-Kβ translocate into the nucleus and initiate the production of PICs (Jaffer et al., 2010). However, the translocation of NF-Kβ in the nucleus and the following production of PICs can be prevented by activated peroxisome proliferator receptor-α and -γ (**PPARα** and **PPARγ**). The PPARα/γ activation is particularly associated to many polyunsaturated fatty acids (**PUFA**), especially omega-3 that, for this reason, have been defined anti-inflammatory (Calder, 2010; Figure 5).

Figure 4. LPS effect on the production of pro-inflammatory cytokines (PICs).

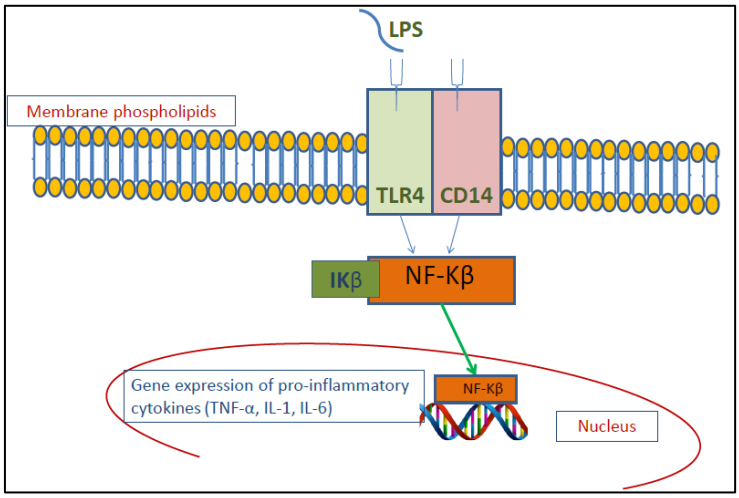
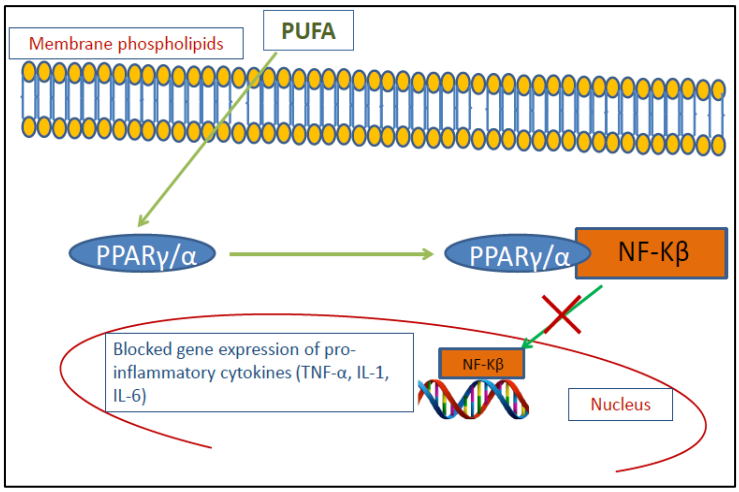


Figure 5. Activated PPARγ and PPARα reduces NF-Kβ translocation into the nucleus.



The high concentration of PICs at the systemic level in addition with the stimulation of the production of other cytokines, may alter the metabolism increasing the catabolism (Bertoni et al., 2015). It was noted that the high concentration of PICs increased the lipolysis and muscle proteolysis processes (Gutierrez et al., 2009; Vaughan et al, 2013). The effects of the PICs may also influence the liver, where they can stimulate the synthesis of acute phase proteins (**APP**) (Jain et al., 2011). Increased systemic concentration of PICs may also affect and stimulate pain in the joints (Rainbow et al., 2012). When PICs concentration is high, body's homeostasis is altered, tissue repair is reduced, the immune system is debilitated and affected individuals are more predisposed to attack by pathogens.

1.4 Conjugated linoleic acids (CLA)

Conjugated linoleic acid is a collective term for a series of conjugated dienoic positional and geometrical isomers of linoleic acid (**LA**; C 18:2, n-6), where, the first digit represents the number of carbon atoms, the second the number of double bonds, and the third the position of the first double bond (Dhiman et al., 2005).

CLA isomers are found naturally in foods, such as: milk and meat from ruminants (Steinhart et al., 2003). In ruminants, CLA are synthesized by ruminal bacteria using LA or (α)-linolenic acid (**aLnA**; C18:3; n-3) as precursors (Kepler et al., 1966). CLA can also be synthesized in the laboratory from LA or from sources high in LA, such as sunflower, safflower, soybean, or corn oils, by a reaction involving alkaline water isomerization 51 and isomerization in propylene glycol (Lehnen et al., 2015). In nature different CLA exist and

until now 28 isomers have been identified (Liu et al., 2011), these are differentiated according to their structure that can vary in cis-cis, cis-trans, trans-cis and trans-trans forms (Rodríguez-Castañedas et al., 2011). However, despite the great number of isomers, the research focuses the attention mainly on two CLA isomers: the cis-9,trans-11 (**c9,t11**; also recognized as rumenic acid) that is the more represented isomer in animal products (80-90%) (Giordano et al., 2011) and the trans-10,cis-12 (**t10,c12**) isomer, particularly important for his effects on health.

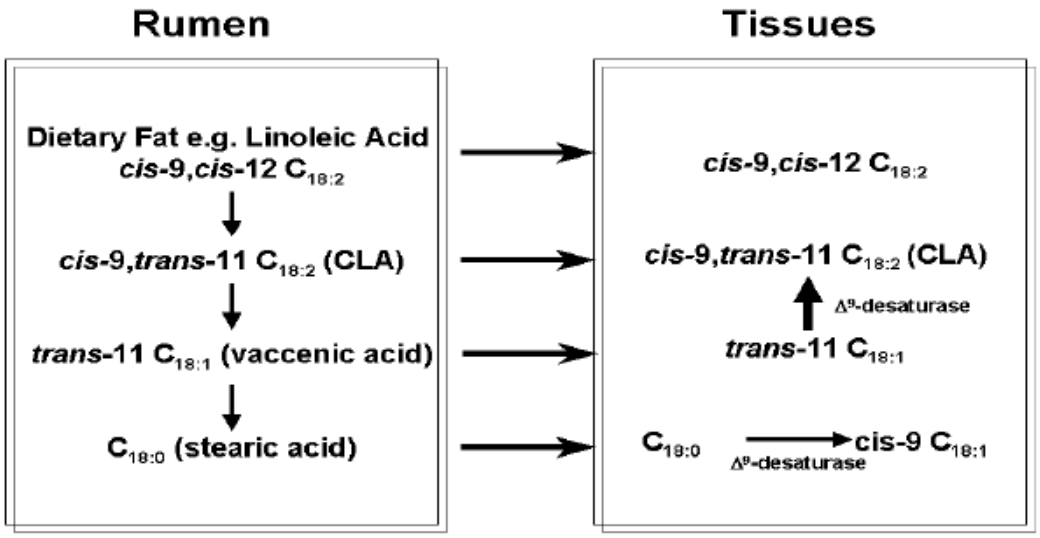
1.4.1 Biosynthesis of CLA in ruminants: rumen biohydrogenation and endogenous synthesis

The CLA in ruminants are formed in separate sites and with different mechanisms: in the rumen during the process of ruminal bio-hydrogenation from LA and aLnA or, in animal tissue through endogenous synthesis starting from an intermediate of biohydrogenation of unsaturated fatty acids.

The composition of forage plants tissue contains a wide range of lipids, that consists essentially of glycolipids and phospholipids (Roca Fernandez and Gonzalez Rodriguez, 2012). The fatty acid profile of these lipids in the grasses is dominated by LA and aLnA, unlike of lipids of seed oils present in concentrates for animals that contain mainly LA and oleic acid (cis-9 C18:1) (Bauman et al. 1999). The suspect that fatty acids were processed in the rumen was when Reiser (1951) noticed that the body fats of ruminants contained less aLnA than horses fed with the same high aLnA diet. To demonstrate this, he incubated aLnA in rumen fluid and showed the formation of trans fatty acids. Shorland et al. (1955) utilizing rumen contents from fistulated sheep grazing pasture, confirmed the existence of trans fatty acid resulting from rumen biohydrogenation. For many years, the only bacterium known to be capable of biohydrogenation was *Butyrivibrio fibrisolvens* (Kepler et al., 1966). However, thanks to the research endeavors many other bacteria have been identified in the rumen that have the ability to hydrogenate unsaturated fatty acids (Harfoot and Hazlewood, 1988). The rumen bacterial species involved in the biohydrogenation process were mainly divided by Kemp and Lander (1984) in 2 groups (A and B). Group A bacteria are able to hydrogenate both LA and aLnA. For LA the biohydrogenation begins with the isomerization of the double bond from position 12 to 11, thus forming the c9,t11 CLA (Figure 6); whereas for the aLnA,

the biohydrogenation leads to the formation of the cis-9,trans-11,cis-15 fatty acid (Nagpal et al., 2007). Vaccenic Acid (**VA**; trans-11 C18:1) is derived from both these products. VA is afterwards utilized by group B bacteria, being their main substrate and from which follows the formation of stearic acid. The rumen pH plays an important role in maintaining environmental conditions of the bacterial flora, this feature influence particularly the synthesis of t10,c12 CLA. Griinari et al. (1998) observed that a low-fiber diet was able to change the fatty acid profile of milk, and trans-10 isomers were the predominant fatty acids. The low-fiber diet, reduces the pH of the rumen and influenced the bacterial cis-9,trans-10 isomerase, with the formation of a t10,c12 double bond. However, the synthesis of t10,c12 CLA is only from LA and not from aLnA (Nagpal et al., 2007).

Figure 6. Ruminal and endogenous synthesis of c9,t11 CLA.



From Bauman et al. (1999).

The synthesis of the CLA may also occur at tissue level, in particular for the isomer c9,t11 CLA. The training starts from the endogenous conversion of VA (Figure 4), the common product of biohydrogenation of both LA and aLna, by desaturase system. The desaturase system is a multi-enzymatic complex that includes NADH-cytochrome b_5 reductase, cytochrome b_5, acyl-CoA synthase, and the Δ^9-desaturase terminal. Between carbons 9 and 10 of the fatty acids Δ^9-desaturase enzyme introduces a cis-double bond, thus forming the c9,t11 CLA (Bauman et al. 1999). The adipose tissue seems to be a major site of endogenous synthesis of c9,t11 CLA in ruminants, but this occurs also in the mammary gland (Bauman et al., 1999; Nagpal et al. 2007).

1.4.2 Effects of CLA

With the discovery of anti-cancer properties of CLA (Pariza, 1979), the interest in these molecules was not limited only to the study of ruminal microbiology but, since then, the CLA have been viewed from another aspect because they were considered as nutraceuticals and therefore molecules that may have beneficial physiological effects on humans and animals.

1.4.2.1 Antineoplastic effect of CLA

The antineoplastic effects of CLA were announced by Pariza et al. (1979). Those authors found in raw ground beef as well as crush the agents with anti-mutagenic action. CLA may prevent the development of cancer in various ways in particular influencing the signal transduction (Comba et al. 2011). Cimini et al. (2005) found that CLA are activators of PPARγ and strongly recommended the use of these FAs such as drugs anti-cancer of brain and as chemotherapeutic agents. The antineoplastic effect of CLA has also been tested on prostate and breast cancer, considered the two most common types of cancer (López-Otín, and Diamandis, 1998).

Ochoa et al. (2004) observed the anti-proliferative effects of c9,t11 and t10,c12 CLA on PC-3 human prostatic carcinoma cell line. Their study showed that both CLA isomers exerted antiproliferative effects, reducing the expression of B-cell lymphoma 2 and induced apoptosis in human prostate cancer cell lines, adjusting also the cell cycle. t10,c12 CLA modulated the apoptosis and cell cycle control, and c9,t11 CLA affected arachidonic acid metabolism.

CLA have been considered effective preventive agents for breast cancer, metastases and some factors concerning this neoplasm (Arab et al., 2016).

Shultz et al. (1992; 1992a) observed *in vitro* on Human MCF-7 breast cancer cells the inhibitory property of CLA on cell growth. Chinnadurai et al. (2008) showed that CLA inhibited malignant cancer induced by dimethylbenz(a)anthracene on female Wistar rats. Banni et al. (2001), noted that CLA provided with diet reduced the number of target epithelial cells in the mammary gland and stimulated the apoptosis in pre-neoplastic cells. Chujo et al.

(2003) in a study on the effect of the growth factor on breast cancer MCF-7 cells, observed that both c9,t11 and t10,c12 CLA can inhibit the proliferation of MCF-7 cells, and results suggested that CLA isomers had separate targets and different mechanisms of actions.

1.4.2.2 Effect on angiogenesis

Some *in vivo* studies have reported that both main CLA isomers may reduce the serum levels of vascular endothelial growth factor (**VEGF**) and Fetal Liver Kinase-1 protein in the mammary gland thus obtaining the angiogenesis control. Masso-Welch et al. (2002) observed that different levels of CLA supplementation (from 1% to 2% of diet), can significantly reduce the angiogenesis, not only by acting on the VEGF, but also by inhibiting the formation of blood vessels functional to carcinogenesis. The hypotheses that could explain the onset of angiogenesis by the CLA are: the reduction of the mobile access network, the inhibition of the micro-capillary connections and the local and systemic VEGF regulation. The administration of t10,c12 CLA isomer also revealed that leptin could be another potential mediator linked to the effect of CLA on angiogenesis.

1.4.2.3 Effect of CLA on diabetes and insulin resistance

The CLA have been proposed as potential therapeutic tools in the management of diabetes type 2. Type 2 diabetes, also called diabetes mellitus, non-insulin-dependent, it's characterized by a variety of metabolic disorders, particularly hyperglycemia, insulin resistance and relative (instead absolute) insulin deficiency (Rodrigues Silva et al., 2014). The use of CLA in rat models has shown that they can improve oral glucose tolerance and delay the onset of disease (Rodrigues Silva et al., 2014). CLA reduces the levels of glucose and insulin in plasma and prevents hyperinsulinemia increasing the levels of plasma adiponectin. However, only the long term treatments can stimulate insulin sensitivity and tolerance glucose, while at the beginning of treatment a negative effect of CLA was observed (Bhattacharya et al., 2006). Clinical studies have shown that t10,c12 CLA can induce hyperinsulinemia in obese individuals, while in other studies was reported that CLA increases insulin sensitivity (Bhattacharya et al., 2006).

1.4.2.4 Antioxidant effect of CLA

Studies in humans and laboratory animals have documented antioxidant effects of CLA. Chinnadurai et al. (2013), Andreoli et al. (2010) and Arab et al. (2006), in cell cultures and in animal models, have shown the protective effects of CLA against oxidative stress and lipid peroxidation. Ha et al. (1990) and MacDonald (2000) through comparative studies have demonstrated that CLA have a higher antioxidant effect than the butilatoidrossitoluene, α-tocopherol and β-carotene. Hanschke et al. (2016) showed that supplementation of 50 g/d and 100 g/d of CLA to lactating German Holstein cows had a marginal antoxidative effect in terms of lipid peroxidation in dairy cows.

1.4.2.5 CLA and inflammatory response

CLA showed an anti-inflammatory effect. Evans et al. (2010) noted that CLA exerted a protective effect on inflammatory bowel disease induced by colorectal cancer in mice; the study suggested that CLA improved colitis and prevented the forming tumors through a PPAR-dependent mechanism. Bhattacharya et al. (2006) reported that CLA in some animal studies induced a negative regulation of the expression of pro-inflammatory genes and activation of apoptosis in atherosclerotic region.

According Dilzer and Park, (2012), the anti-inflammatory effect of CLA could result from different mechanisms of action, such as the reduction of eicosanoids production, the increase in anti-inflammatory response mediated by PPAR with the resulting inactivation of NF-κβ and the reduced expression of PICs (TNF-α; IL-1, IL-6).

1.4.2.6 Effect of CLA on bone growth and metabolism

The local growth factors such as the insulin-like growth factor-1 (**IGF-1**), prostaglandin E2 (**PGE2**), the cytokines IL-1 and TNF-α, affect the cells activity responsible of bone metabolism. The CLA supplemented to a level of 1% w/w of the diet depressed the *in vivo* synthesis of PGE2 and IGF-1 serum and the rate of bone formation in male rats. However, CLA isomers were responsible in a dose-dependent manner of collagen synthesis and of growth plate in chondrocytes in primary cultures. The type and the amount of CLA

isomers used in the experiments as well as the ratio of omega-6 to omega-3 fatty acids in the diet influenced differently the bone metabolism in rats (Rodrigues Silva et al., 2014).

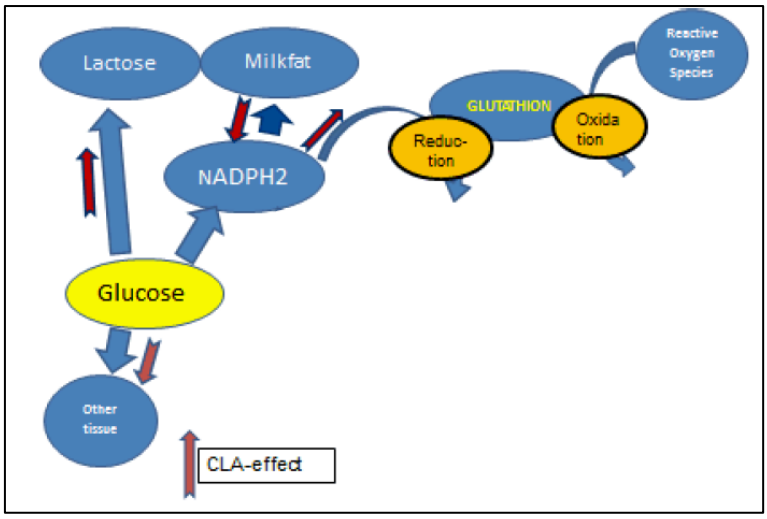
1.4.2.7 Effects of CLA on lipid metabolism

Among the portentous effects of CLA is of considerable interest their ability to decrease body fat without affecting total body mass. The capability of the CLA to affect lipid metabolism and change the body condition was observed in several animal models. West et al. (1998) have characterized the metabolic effects of CLA on male AKR/J mice fed with a high-fat (45 kcal%) or low-fat (15 kcal%) diet with or without CLA (2.46 mg/kcal; 1.2 and 1.0% by weight in high- and low-fat diets, respectively) for 6 wk. Those authors observed that CLA significantly reduced energy intake, growth rate, adipose depot weight, and carcass lipid and protein content independent of diet composition. The reduction of adipose depot weight ranged from 43 to 88%. CLA significantly increased metabolic rate and decreased the nighttime respiratory quotient. These findings demonstrate that CLA reduce body fat by several mechanisms, including a reduced energy intake, increased metabolic rate, and a shift in the nocturnal fuel mix.

A study on pigs fed with CLA showed that treated animals developed less body fat and improved feed conversion efficiency (Dugan et al. 1997). Another study of Ostrowska et al. (1999) showed that CLA supplements increased lean tissue deposition and decreased fat deposition in pigs. The *in vitro* and *in vivo* studies reported that among the different CLA isomers, only the t10,c12 CLA was the responsible for the inhibition of accumulation of body fat and changes of body composition. The c9,t11 CLA did not have any effects. The mentioned information indicated that c9,t11 CLA was active in enhancing bodyweight gain and also appeared to enhance feed efficiency in weanling mice (Rodrigues Silva et al., 2014). The t10,c12 CLA was able to inhibit the growth by altering the activities of lipogenic and oxidative gene targets transcriptionally controlled by sterol regulatory element-binding proteins-1 c, and PPARγ (Belury, 2002). Influence of CLA on fat synthesis was also observed in dairy cow. Several studies on dairy cows showed that t10,c12 CLA induces milk fat depression. In particular, t10,c12 CLA inhibited milk fat synthesis in the mammary gland inducing an energy saving and increasing milk yield (Bauman et al., 2008). In fact, the reduced *de novo* fatty acids synthesis would induce an increased availability of NADPH as

well as glucose. NADPH would be required for other biological processes such as for example oxidative processes and glucose would be indirectly responsible for the increased production of milk (Figure 7).

Figure 7. Link between CLA, milk fat, glucose and oxidative process in dairy cow



1.5 Essential fatty acids (EFAs)

Omega-6 and omega-3 are called essential because mammals cannot produce them by themselves and are obliged to hire them through the diet, to keep the body in good health. Rather, the synthesis of EFAs occurs in plants thanks to the presence of Δ-12 and Δ-15 desaturase enzymes (Lee et al., 2016).

Essential fatty acids are subdivided into two distinct classes: omega-6 and omega-3 fatty acids. The main difference between omega-6 and omega-3 fatty acids is based on the location of the first double bond, counting from the methyl end of the fatty acid molecule. In the omega-6 fatty acids, the first double bond is between the 6^{th} and 7^{th} carbon atoms and for the omega-3 fatty acids the first double bond is between the 3^{rd} and 4^{th} carbon atoms (Hussein et al. 2013). The representative of the omega-6 fatty acids is the LA, while the aLnA represents the omega-3 fatty acids. LA is very abundant in nature, is present in the seeds of many plants and plant oils with the exception of coconut, cocoa, and palm. Whereas aLnA, is mainly found in the chloroplasts of green leafy vegetables, in fish, and in the seeds of flax, turnips, chia, perilla and in walnuts (Simopoulos, 2016; Table 1).

Table 1. PUFA content of dietary components

Fat type	LA	aLnA	AA	EPA+DHA
Saturated				
Lard	8600	1000	1070	
Butterfat	2300	1400		
Coconut oil	1400			
Beef tallow	80			
Unsatured				
a) Monounsaturated				
Peanut oil	23900			
Pecans	20600	1000		
Almonds	9860	260		
Olive oil	8000	950		
Avocado	1970			
b) Polynsaturated				
Omoga-6				
Safflower oil	74000	470		
Sunflower oil	60200	500		
Soybean oil	53400	7600		
Corn oil	50000	900		
Cotton seed oil	47800	1000		
Walnut	34100	6800	590	
Brazil nut	24900			
Omega-3				
Linseed oil	13400	55300		
Canola oil	19100	8600		
Salmon	440	550	300	1200
Tuna	260	270	280	400
Herring	150	62	37	1700
Trout	74		30	500
Cod	4	2	3	300

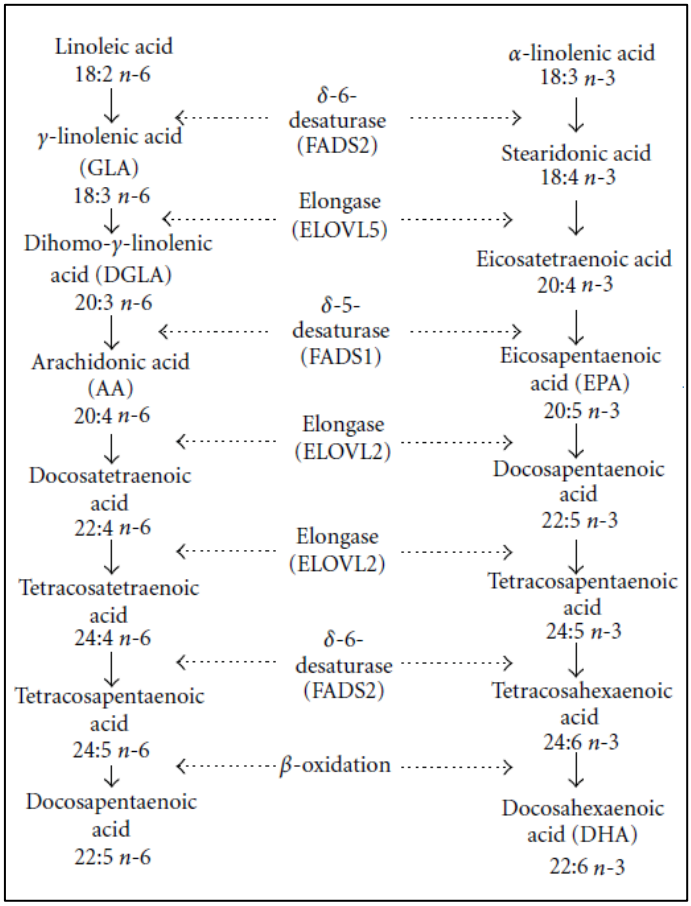
Data are expressed as mg/100g edible portion. Data are elaborated from Patterson et al. (2012).

LA= Linoleic acid; aLnA = α-Linolenic acid; gLnA = γ-linolenic acid; Arachidonic acid= AA; Eicosapentaenoic acid=EPA ; Docosahexaenoic acid= DHA

Both LA and aLnA assumed by mammals are metabolized to longer-chain fatty acids of 20 and 22 carbon atoms. LA is converted to arachidonic acid (**AA**; 20:4n-6) via (γ)-linolenic acid (**gLna**; 18:3n-6) and dihomo-γ-linolenic acid (**DGLA**, 20:3n-6). By the same set of enzymes, aLnA can be converted to eicosapentaenoic acid (**EPA**; 20:5n-3) and docosahexaenoic acid (**DHA**, 22:6n-3). The process is made possible by increasing the chain

length and the degree of unsaturation, adding extra double bonds to the carboxyl end of the fatty acid molecule (Simopoulos, 2008; Figure 8).

Figure 8. Metabolism of omega-6 and omega-3 in the body.



From Patterson et al. (2012), modified

From mammals LA and aLnA are respectively converted in AA, EPA and DHA, through a slow process. Moreover, among the 2 distinct families of fatty acids exist a competition for the desaturation enzymes. The Δ-4 and Δ-6 desaturase enzymes prefer omega-3 to omega-6 FAs, even if a high LA intake interferes with the desaturation and elongation of aLnA. The conversion between the two families of fatty acids is not possible for the lack of conversion enzymes (Simopoulos, 2008). These fatty acids together with their derivatives are found within the cell membranes of mammals and birds as in triglycerides, in cholesterol esters, and in small amounts also in the phospholipids. the types of fatty acids in the diet therefore modify the cellular fatty pool, in particular the changes are more resentful in

the membranes of platelets, erythrocytes, neutrophils, monocytes, and liver cells (Hussein, 2013; Simopoulos, 2008).

1.5.1 Effects of EFAs on inflammatory process

When begins an inflammatory response, the membrane phospholipases are activated and the injured cell releases fatty acids to 20 carbon atoms from phospholipid membrane (Calder, 2013). Precisely, from these fatty acids are then formed different types of eicosanoids: prostaglandins, leukotrienes, prostacyclins and thromboxanes (Karaulov et al., 1986). The eicosanoids are immunomodulatory molecules which exercise a local hormone-like effect. (Bhathena , 2000; De Pablo and De Cienfuegos, 2000). These substances are not stored in the body, and are synthesized according to the requirement using polyunsaturated fatty acids present in membrane phospholipids. The activity of the two-metabolic enzyme systems, such as cyclooxygenase (**COX**) and lipoxygenase, together with the availability of fatty acid precursors affect the amount and type of eicosanoid synthesized (Ding et al., 2003). The compounds obtained by the omega-6 fatty acids are much more pro-inflammatory than those obtained from the metabolism of omega-3 (Wall et al., 2010) and also, the eicosanoids from AA are formed in greater quantities than those formed from omega-3 fatty acids, specifically EPA (Simopolous, 2008). The influence of dietary PUFA on phospholipid composition influence the inflammatory response of the cells (Sordillo and Raphael, 2013).

AA is released from membrane phospholipids by phospholipase A_2 (**PLA_2**) and, once free, is used as a substrate by enzymes that synthesize eicosanoids (Balsinde et al., 2002). The metabolic pathway of AA that uses the enzyme COX leads to the synthesis of series 2 prostaglandin (**PG**) and thromboxane. The COX enzymes are divided in two isoforms: COX-1 and COX-2. Both are responsible for the production of PG. COX-1 is important for homeostatic function, such as, maintaining the integrity of the gastric mucosa and mediating normal platelet function, instead COX-2 is in particular overexpressed during inflammation (Crofford, 1997; Ricciotti and FitzGerald, 2011). With regard to the leukotrienes, in particular of the B_4 series, they increase the vascular permeability, are vasoconstrictors, increase local blood flow, induce the release of lysosomal enzymes and stimulate the production of interleukins (Back et al., 2004; Björk et al., 1982; Hafstrom et al., 1981; Larkin et al., 1995;

Rola-Pleszczynski and Stankova, 1992). For these reasons, the leukotrienes are considered natural pro-inflammatory mediators (Busse, 1998).

The excessive pro-inflammatory effect of AA derivatives is mitigated by omega-3 fatty acids, which modify the inflammatory process (Calder, 2010). The increased amount of omega-3 in the diet than omega-6, leads to a decreased percentage of AA in inflammatory cell membranes and also of pro-inflammatory agents (Simopolous, 2002). Omega-3 fatty acids compete with AA using the same metabolic pathways, providing the substrate for the same enzymes, therefore, the contrast action is not limited only to a simple reduction of the available substrate. From derivatives of omega-3, the 5-series of leukotriene and the 3-series of PG and thromboxane are produced (Patterson et al., 2012). The 5-series leukotrienes and the 3-series prostaglandins and thromboxanes are considered less inflammatory or even anti-inflammatory compared to eicosanoid derived from AA (Robinson and Stone., 2006). EPA and DHA can inhibit the production of classic inflammatory cytokines, such as TNF-α, IL-1, IL-6 and Interleukin-8 (**IL-8**) (Borges et al., 2014; Calder, 2006). Many studies have shown that consumption of omega-3 or its derivatives, reduces the incidence of diseases and chronic inflammations (Dominiguez, 2013).

Some of the most chronic diseases in humans are characterized by an exaggerated inflammatory response and of an excessive production of cytokines and inflammatory eicosanoids. Among these chronic diseases are included the rheumatoid arthritis, Alzheimer's disease, cardiovascular disease and atherosclerosis and *inflammatory bowel disease* (Patterson et al., 2012). Furthermore, it was showed that diet supplemented with omega-3 fatty acids during pregnancy is essentially important as they are critical building blocks of fetal brain and retina and can also play a role in influencing the length of gestation and in preventing perinatal depression (Coletta, 2010).

1.5.2 Effects of EFAs on cardiovascular diseases

The importance of EFAs was already known in the late 1970s when epidemiological studies in this period revealed that Greenland Eskimos populations consumed a high seafood diet and had low rates of coronary heart disease than Western populations (O'Keefe and Harris, 2000).

The present Western diet is poor in aLnA and its derivatives, and the ratio is in favor of omega-6 rather than omega-3 (Simopolous, 2008). The high amount of LA determines a

greater quantity of AA and, in turn, an increased production of pro-inflammatory eicosanoids derivatives. Diet rich in LA changes the physiological been to one that is pro-thrombotic and pro-aggregatory, with an increased blood viscosity, vasoconstriction and vasospasm (Simopoulos, 2016). Increasing dietary omega-3 fatty acids increases the same fatty acids and derivatives in membrane phospholipids, which in turn may influence the fluidity and permeability of the plasma membrane (Carughi et al., 2010). From these observation, the beneficial effects of aLnA and his derivatives on health were highlighted. Omega-3 were recognized to have antithrombotic, anti-aterogenic, antiarrhythmic effects (DE Caterina and Zampolli, 2006) and to mediate the regulation of the pressure, and the level of blood cholesterol and triglycerides (Bradberry and Hilleman, 2013). A regular consumption of sources of omega-3 fatty acids is able to reduce the risk of coronary death and total mortality (Mohebi-Nejad and Bikdeli, 2014). Omega-3 fatty acids decrease platelet aggregation (Gao et al., 2013) resulting in a modest prolongation of bleeding times, this would result in a lower thrombus formation, probably related to the ability of omega-3 in reducing platelet signaling, and also to alter the processing of thrombin precursor proteins (Larson et al., 2013). The effects of the increased amount of derived omega-3 thromboxane change the types of products than those formed from AA, with the relative modification of the biological activity. The thromboxane A3, synthesized from EPA, are less powerful and with reduced vasoconstrictor and aggregating effects than of thromboxane A2 (derived from AA) (Wardhana et al., 2013). Another important biological function of omega-3 is the reduction of plasma triglycerides. The triglycerides lowering effect of EPA + DHA has been demonstrated in numerous studies: 3-4 g/day of omega-3 decreases plasma triglycerides by about 30% (16-45% range) (Shearer et al., 2012). The evidence suggests that omega-3 fatty acids reduce the synthesis and secretion of very-low-density lipoprotein (**VLDL**) particles, and increase triglycerides removal from VLDL and chylomicron particles through the up-regulation of enzymes, such as lipoprotein lipase (Bays et al., 2008).

1.6 Objectives and Outline of this Thesis

The objectives of this thesis were (1) to investigate the *in vitro* effects of t10,c12 and c9,t11 CLA isomers on the cellular antioxidant response of bovine mammary cells epithelial cells (**BME-UV1**) examining whether CLA isomers could play a role in cell protection against the oxidative stress and studying the molecular mechanism involved; (2) to investigate and compare the antioxidant and anti-inflammatory effect between CLA and other EFAs in BME-UV1 cells, evaluating the potential protection of different FAs against oxidative stress, their protective efficacy against inflammatory status through the expression of pro and anti-inflammatory cytokines.

The outline of this thesis is as follows:

Chapter 1 covers the assessment of the antioxidant effect of CLA isomers administered to BME-UV1 cells, evaluating the CLA uptake by the cells, the antioxidant cellular response of the main oxidative stress markers and their protective efficacy against H_2O_2-induced oxidative stress.

Chapter 2 covers the comparison of the antioxidant effect between CLA isomers and other EFAs administered to BME-UV1 cells, evaluating the antioxidant cellular response of the main oxidative stress markers and their protective efficacy against H_2O_2-induced oxidative stress.

Chapter 3 covers the comparison of the anti-inflammatory effects of CLA isomers and other EFAs administered to BME-UV1 cells treated with LPS, evaluating the gene expression of pro and anti-inflammatory cytokines.

1.7 References

Abuelo, A., J. Hernández, J. L. Benedito, and C. Castillo. 2015. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. J. Anim. Physiol. Anim. Nutr. (Berl). 99:1003–1016. doi:10.1111/jpn.12273.

Ahmad, R., A. K. Tripathi, P. Tripathi, S. Singh, R. Singh, and R. K. Singh. 2008. Malondialdehyde and protein carbonyl as biomarkers for oxidative stress and disease progression in patients with chronic myeloid leukemia. In Vivo 22:525–528.

Andreoli, M. F., P. G. Illesca, M. A. González, and C. A. Bernal. 2010. Conjugated Linoleic Acid Reduces Hepatic Steatosis and Restores Liver Triacylglycerol Secretion and the Fatty Acid Profile During Protein Repletion in Rats. Lipids 45:1035–1045. doi:10.1007/s11745-010-3466-3474.

Arab, A., S. A. Akbarian, R. Ghiyasvand, and M. Miraghajani. 2016. The effects of conjugated linoleic acids on breast cancer: A systematic review. Adv. Biomed. Res. 5:115. doi:10.4103/2277-9175.185573.

Arab, K., Rossary, A., Soulere, L. and J. P. Steghens. 2006. Conjugated linoleic acid, unlike other unsaturated fatty acids, strongly induces glutathione synthesis without any lipoperoxidation. Br. J. Nutr. 96:811-819.

Arango Duque, G., and A. Descoteaux. 2014. Macrophage cytokines: involvement in immunity and infectious diseases. Front. Immunol. 5:491. doi:10.3389/fimmu.2014.00491.

Ayala, A., M. F. Muñoz, and S. Argüelles. 2014. Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. Oxid. Med. Cell. Longev. 2014:1–31. doi:10.1155/2014/360438.

Back, M., H. Qiu, J. Z. Haeggström, and K. Sakata. 2004. Leukotriene B4 is an indirectly acting vasoconstrictor in guinea pig aorta via an inducible type of BLT receptor. A. J. P. Hear. Circ. Physiol. 287:H419–H424. doi:10.1152/ajpheart.00699.2003.

Balsinde, J., M. V. Winstead, and E. A. Dennis. 2002. Phospholipase A_2 regulation of

arachidonic acid mobilization. FEBS Lett. 531:2–6. doi:10.1016/S0014-5793(02)03413-0.

Banni, S., E. Angioni, E. Murru, G. Carta, M. Paola Melis, D. Bauman, Y. Dong, and C. Ip. 2001. Vaccenic Acid Feeding Increases Tissue Levels of Conjugated Linoleic Acid and Suppresses Development of Premalignant Lesions in Rat Mammary Gland. Nutr. Cancer 41:91–97. doi:10.1080/01635581.2001.9680617.

Barelli, S., G. Canellini, L. Thadikkaran, D. Crettaz, M. Quadroni, J. S. Rossier, J.-D. Tissot, and N. Lion. 2008. Oxidation of proteins: Basic principles and perspectives for blood proteomics. PROTEOMICS – Clin. Appl. 2:142–157. doi:10.1002/prca.200780009.

Bays, H. E., A. P. Tighe, R. Sadovsky, and M. H. Davidson. 2008. Prescription omega-3 fatty acids and their lipid effects: physiologic mechanisms of action and clinical implications. Expert Rev. Cardiovasc. Ther. 6:391–409. doi:10.1586/14779072.6.3.391.

Baumgard L. H, J. K. Kay, C. E. Moore, J. R. Roche, M. J. Vanbaale, and R. P. Rhoads. 2005. Regulating milk fat synthesis: potential on-farm applications of basic science. Proc. New Zeal. Soc. Anim. Prod. 65:33–39.

Belury, M. A. 2002. DIETARY C ONJUGATED LINOLEIC ACID IN HEALTH: Physiological Effects and Mechanisms of Action. Annu. Rev. Nutr. 22:505–531. doi:10.1146/annurev.nutr.22.021302.121842.

Berlett, B. S., and E. R. Stadtman. 1997. Protein oxidation in aging, disease, and oxidative stress. J. Biol. Chem. 272(33):20313–20316.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2002. Markers of Oxidative Status in Plasma and Erythrocytes of Transition Dairy Cows During Hot Season. J. Dairy Sci. 85:2173–2179. doi:10.3168/jds.S0022-0302(02)74296-3.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of Body Condition Score on Relationships Between Metabolic Status and Oxidative Stress in Periparturient Dairy Cows. J. Dairy Sci. 88:2017–2026. doi:10.3168/jds.S0022-0302(05)72878-2.

Bertoni, G., A. Minuti, and E. Trevisi. 2015. Immune system, inflammation and nutrition in dairy cattle. Anim. Prod. Sci. 55:943-949. doi:10.1071/AN14863.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008. Effects of Inflammatory Conditions on Liver Activity in Puerperium Period and Consequences for Performance in Dairy Cows. J. Dairy Sci. 91:3300–3310. doi:10.3168/jds.2008-0995.

Betteridge, D.J. 2000. What is oxidative stress?. Metabolism. 49:3–8.

Bhathena S. J. 2000. Relationship between fatty acids and the endocrine system. Biofactors 13:35–39.

Bhattacharya, A., J. Banu, M. Rahman, J. Causey, and G. Fernandes. 2006. Biological effects of conjugated linoleic acids in health and disease. J. Nutr. Biochem. 17:789–810. doi:10.1016/j.jnutbio.2006.02.009.

Birben, E., U. M. Sahiner, C. Sackesen, S. Erzurum, and O. Kalayci. 2012. Oxidative stress and antioxidant defense.. World Allergy Organ. J. 5:9–19. doi:10.1097/WOX.0b013e3182439613.

Björk, J, Hedqvist, P., and K. E. Arfors. 1982. Increase in vascular permeability induced by leukotriene B4 and the role of polymorphonuclear leukocytes. Inflammation. 6:2, 189-200.

Bradberry, J. C., and D. E. Hilleman. 2013. Overview of omega-3 Fatty Acid therapies. P T 38:681–691.

Borges, M. C., F. de M. M. Santos, R. W. Telles, M. I. T. D. Correia, C. C. D. Lanna, M. C. Borges, F. de M. M. Santos, R. W. Telles, M. I. T. D. Correia, and C.C.D. Lanna. 2014. Ácidos graxos poli-insaturados ômega-3 e lúpus eritematoso sistêmico: o que sabemos? Rev. Bras. Reumatol. 54:459–466. doi:10.1016/j.rbr.2013.12.002.

BUSSE, W. W. 1998. Leukotrienes and Inflammation. Am. J. Respir. Crit. Care Med. 157:S210–S213. doi:10.1164/ajrccm.157.6.mar-1.

Calder, P. C. 2006. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am. J. Clin. Nutr. 83:1505S–1519S.

Calder, P. C. 2010. Omega-3 fatty acids and inflammatory processes. Nutrients 2:355–374. doi:10.3390/nu2030355.

Calder, P. C. 2013. Omega-3 polyunsaturated fatty acids and inflammatory processes:

nutrition or pharmacology? Br. J. Clin. Pharmacol. 75:645–662. doi:10.1111/j.1365-2125.2012.04374.x.

Carughi, A., Liem, Huynh, T. and D. Perelman. 2010. Effect of omega-3 fatty acid supplementation on indicators of membrane fluidity. The FASEB J. 24(1):912-939

Castillo, C., J. Hernández, I. Valverde, V. Pereira, J. Sotillo, M. L. Alonso, and J. L. Benedito. 2006. Plasma malonaldehyde (MDA) and total antioxidant status (TAS) during lactation in dairy cows. Res. Vet. Sci. 80:133–139. doi:10.1016/j.rvsc.2005.06.003.

Celi, P. 2010. The role of oxidative stress in small ruminants' health and production. Rev. Bras. Zootec. 39:348–363. doi:10.1590/S1516-35982010001300038.

Chinnadurai, K., A. K. Tyagi, and P. Krishnamoorthy. 2008. Influence of conjugated linoleic acid enriched ghee feeding on cancer incidence and histopathological changes in 7,12-dimethyl- benz[a]anthracene induced mammary gland carcinogenesis in rats. Vet. Arh. 78:511–520.

Chinnadurai, K., H. K. Kanwal, A. K. Tyagi, C. Stanton, and P. Ross. 2013. High conjugated linoleic acid enriched ghee (clarified butter) increases the antioxidant and antiatherogenic potency in female Wistar rats. Lipids Health Dis. 12:121. doi:10.1186/1476-511X-12-121.

Chujo, H., M. Yamasaki, S. Nou, N. Koyanagi, H. Tachibana, and K. Yamada. 2003. Effect of conjugated linoleic acid isomers on growth factor-induced proliferation of human breast cancer cells. Cancer Lett. 202:81–87.

Ciaccia L. Fundamentals of inflammation. The Yale J. Bio. Med. 2011; 84(1):64-65.

Cimini, A., L. Cristiano, S. Colafarina, E. Benedetti, S. Di Loreto, C. Festuccia, F. Amicarelli, R. A. Canuto, and M. P. Cerù. 2005. PPARγ-dependent effects of conjugated linoleic acid on the human glioblastoma cell line (ADF). Int. J. Cancer 117:923–933. doi:10.1002/ijc.21272.

Coletta, J. M., S. J. Bell, and A. S. Roman. 2010. Omega-3 Fatty acids and pregnancy. Rev. Obstet. Gynecol. 3:163–171

Comba, A., Y.-H. Lin, A. R. Eynard, M. A. Valentich, M. E. Fernandez-Zapico, and M. E.

Pasqualini. 2011. Basic aspects of tumor cell fatty acid-regulated signaling and transcription factors. Cancer Metastasis Rev. 30:325–342. doi:10.1007/s10555-011-9308-x.

Contreras, G. A., and L. M. Sordillo. 2011. Lipid mobilization and inflammatory responses during the transition period of dairy cows. Comp. Immunol. Microbiol. Infect. Dis. 34:281–289. doi:10.1016/j.cimid.2011.01.004.

Costa, V., A. Quintanilha, and P. Moradas-Ferreira. 2007. Protein oxidation, repair mechanisms and proteolysis in Saccharomyces cerevisiae. IUBMB Life 59:293–298. doi:10.1080/15216540701225958.

Cuadrado, A., P. Moreno-Murciano, and J. Pedraza-Chaverri. 2009. The transcription factor Nrf2 as a new therapeutic target in Parkinson's disease. Expert Opin. Ther. Targets 13:319–329. doi:10.1517/13543780802716501.

Dalle-Donne, I., R. Rossi, R. Colombo, D. Giustarini, and A. Milzani. 2006. Biomarkers of oxidative damage in human disease. Clin. Chem. 52:601–623. doi:10.1373/clinchem.2005.061408.

David, S. S., V. L. O'Shea, and S. Kundu. 2007. Base-excision repair of oxidative DNA damage. Nature 447:941–950. doi:10.1038/nature05978.

Davies, M. J. 2016. Protein oxidation and peroxidation. Biochem. J. 473:805–825. doi:10.1042/BJ20151227.

DE Caterina, R., and A. Zampolli. 2006. Antiatherogenic effects of n-3 fatty acids - evidence and mechanisms. Heart Int. 2:141. doi:10.4081/hi.2006.141.

De Pablo, M. A., and G. A. De Cienfuegos. 2000. Modulatory effects of dietary lipids on immune system functions. Immunol. Cell Biol. 78:31–39. doi:10.1046/j.1440-1711.2000.00875.x.

Dhiman, T. R., S.-H. Nam, and A. L. Ure. 2005. Factors Affecting Conjugated Linoleic Acid Content in Milk and Meat. Crit. Rev. Food Sci. Nutr. 45:463–482. doi:10.1080/10408390591034463.

Dilzer, A., and Y. Park. 2012. Implication of Conjugated Linoleic Acid (CLA) in Human

Health. Crit. Rev. Food Sci. Nutr. 52:488–513. doi:10.1080/10408398.2010.501409.

Ding, X.-Z., R. Hennig, and T. E. Adrian. 2003. Lipoxygenase and cyclooxygenase metabolism: new insights in treatment and chemoprevention of pancreatic cancer.. Mol. Cancer 2:10. doi:10.1186/1476-4598-2-10

Dominguez, H. (Ed.). (2013). Functional ingredients from algae for foods and nutraceuticals. Elsevier. Pag 765

Drackley, J. K. 1999. Biology of dairy cows during the transition period: the final frontier? J. Dairy Sci. 82:2259–2273. doi:10.3168/jds.S0022-0302(99)75474-3.

Dugan, M. E. R., J. L. Aalhus, A. L. Schaefer, and J. K. G. Kramer. 1997. The effect of conjugated linoleic acid on fat to lean repartitioning and feed conversion in pigs. Can. J. Anim. Sci. 77:723–725. doi:10.4141/A97-084.

Espinosa-Diez, C., V. Miguel, D. Mennerich, T. Kietzmann, P. Sánchez-Pérez, S. Cadenas, and S. Lamas. 2015. Antioxidant responses and cellular adjustments to oxidative stress. Redox Biol. 6:183–197. doi:10.1016/j.redox.2015.07.008.

Evans, N. P., S. A. Misyak, E. M. Schmelz, A. J. Guri, R. Hontecillas, and J. Bassaganya-Riera. 2010. Conjugated linoleic acid ameliorates inflammation-induced colorectal cancer in mice through activation of PPARgamma. J. Nutr. 140:515–521. doi:10.3945/jn.109.115642.

Gao, L., J. Cao, Q. Mao, X. Lu, X. Zhou, and L. Fan. 2013. Influence of omega-3 polyunsaturated fatty acid-supplementation on platelet aggregation in humans: A meta-analysis of randomized controlled trials. Atherosclerosis 226:328–334. doi:10.1016/j.atherosclerosis.2012.10.056.

Giordano, E., S. Banni, and L. Quadro. 2011. A single dose of c9,t11 or t10,c12 conjugated linoleic acid isomers perturbs vitamin A metabolism in mice. Nutr. Res. 31:855–862. doi:10.1016/j.nutres.2011.09.013.

Giovannini, C., C. Filesi, M. D'Archivio, B. Scazzocchio, C. Santangelo, and R. Masella. Polifenoli e difese antiossidanti endogene: effetti sul glutatione e sugli enzimi ad esso correlati. Ann. Ist. Superiore Sanità 42(3):336–347.

Griinari, J. M., D. A. Dwyer, M. A. McGuire, D. E. Bauman, D. L. Palmquist, and K. V. V. Nurmela. 1998. Trans-Octadecenoic Acids and Milk Fat Depression in Lactating Dairy Cows. J. Dairy Sci. 81:1251–1261. doi:10.3168/jds.S0022-0302(98)75686-3.

Grimble, R. F. 1990. Nutrition and cytokine action. Nutr. Res. Rev. 3:193. doi:10.1079/NRR19900012.

Grummer, R. R. 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. J. Dairy Sci. 76:3882–3896. doi:10.3168/jds.S0022-0302(93)77729-2.

Guilford, F., and J. Hope. 2014. Deficient glutathione in the pathophysiology of mycotoxin-related Illness. Toxins (Basel). 6:608–623. doi:10.3390/toxins6020608.

Gutierrez, D.A., M.J. Puglisi, and A.H. Hasty. 2009. Impact of increased adipose tissue mass on inflammation, insulin resistance, and dyslipidemia.. Curr. Diab. Rep. 9:26–32.

Ha, Y.L., Storkson, J. and Pariza, M. W. 1990. Inhibition of benz(a)pyreneinduced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. Cancer Res. 50:1097–1101.

Hafstrom, I., J. Palmblad, C. L. Malmsten, O. Rådmark, and B. Samuelsson. 1981. Leukotriene B4 — a stereospecific stimulator for release of lysosomal enzymes from neutrophils. FEBS Lett. 130:146–148. doi:10.1016/0014-5793(81)80684-9.

Halliwell, B., and S. Chirico. 1993. Lipid peroxidation: its mechanism, measurement, and significance.. Am. J. Clin. Nutr. 57:715S–725S.

Halliwell, B.; and J. M. C. Gutteridge. 1989. Free radicals in biology and medicine. Oxford: Oxford University Press,. 543p.

Hanschke, N., M. Kankofer, L. Ruda, M. Höltershinken, U. Meyer, J. Frank, S. Dänicke, and J. Rehage. 2016. The effect of conjugated linoleic acid supplements on oxidative and antioxidative status of dairy cows. J. Dairy Sci. 99:8090–8102. doi:10.3168/jds.2015-10685.

Hato, T., and P. C. Dagher. 2015. How the Innate Immune System Senses Trouble and Causes Trouble. Clin. J. Am. Soc. Nephrol. 10:1459–1469. doi:10.2215/CJN.04680514.

Jaffer, U., R. G. Wade, and T. Gourlay. 2010. Cytokines in the systemic inflammatory

response syndrome: a review. HSR Proc. Intensive Care Cardiovasc. Anesth. 2:161–175.

Jain, S., V. Gautam, and S. Naseem. 2011. Acute-phase proteins: As diagnostic tool.. J. Pharm. Bioallied Sci. 3:118–127. doi:10.4103/0975-7406.76489.

Johnson, A. R., J. J. Milner, and L. Makowski. 2012. The inflammation highway: metabolism accelerates inflammatory traffic in obesity.. Immunol. Rev. 249:218–238. doi:10.1111/j.1600-065X.2012.01151.x.

Kakhlon, O., and Z. I. Cabantchik. 2002. The labile iron pool: characterization, measurement, and participation in cellular processes. Free Radic. Biol. Med. 33:1037–1046.

Karaulov, A. V, V. P. Silvestrov, V. D. Pomoĭnetskiĭ, V. N. Zakharov, and V. P. Masenko. 1986. [Role of the eicosanoids: prostaglandins, prostacyclin, thromboxane and leukotrienes in the pathogenesis of bronchial asthma and other lung diseases]. Ter. Arkh. 58:123–130.

KEMP, P., and D. J. LANDER. 1984. Hydrogenation in vitro of linolenic acid to stearic acid by mixed cultures of pure strains of rumen bacteria. Microbiology 130:527–533. doi:10.1099/00221287-130-3-527.

Kepler, C. R., K. P. Hirons, J. J. Mc Neill, and S. B. Tove. 1966. Intermediates and products of the biohydrogenation of linoleic acid by Butyrinvibrio fibrisolvens.. J. Biol. Chem. 241:1350–1354.

Land, W. G. 2015. The role of damage-associated molecular patterns in human diseases: Part I - Promoting inflammation and immunity. Sultan Qaboos Univ. Med. J. 15:e9–e21.

Larkin, S. W., L. Fraser, H. J. Showell, T. J. Williams, and J. B. Warren. 1995. Prolonged microvascular vasodilation induced by leukotriene B4 in human skin is cyclooxygenase independent.. J. Pharmacol. Exp. Ther. 272:392–398.

Larson, M. K., G. W. Tormoen, L. J. Weaver, K. J. Luepke, I. A. Patel, C. E. Hjelmen, N. M. Ensz, L. S. McComas, and O. J. T. McCarty. 2013. Exogenous modification of platelet membranes with the omega-3 fatty acids EPA and DHA reduces platelet procoagulant activity and thrombus formation. Am. J. Physiol. Cell Physiol. 304:C273- C279. doi:10.1152/ajpcell.00174.2012.

Lee, J. M., H. Lee, S. Kang, and W. J. Park. 2016. Fatty Acid Desaturases, Polyunsaturated Fatty Acid Regulation, and Biotechnological Advances.. Nutrients 8. doi:10.3390/nu8010023.

Lehnen, T. E., M. R. da Silva, A. Camacho, A. Marcadenti, and A. M. Lehnen. 2015. A review on effects of conjugated linoleic fatty acid (CLA) upon body composition and energetic metabolism.. J. Int. Soc. Sports Nutr. 12:36. doi:10.1186/s12970-015-0097-4.

Leroy, J., R. Sturmey, V. Van Hoeck, J. De Bie, P. McKeegan, and P. Bols. 2014. Dietary fat supplementation and the consequences for oocyte and embryo quality: hype or significant benefit for dairy cow reproduction?. Reprod. Domest. Anim. 49:353–361. doi:10.1111/rda.12308.

Liu, P., S.-R. Shen, H. Ruan, Q. Zhou, L.-L. Ma, and G.-Q. He. 2011. Production of conjugated linoleic acids by Lactobacillus plantarum strains isolated from naturally fermented Chinese pickles.. J. Zhejiang Univ. Sci. B 12:923–930. doi:10.1631/jzus.B1100072.

Lobo, V., A. Patil, A. Phatak, and N. Chandra. 2010. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn. Rev. 4:118. doi:10.4103/0973-7847.70902.

López-Otín, C., and E. P. Diamandis. 1998. Breast and Prostate Cancer: An analysis of common epidemiological, genetic, and biochemical features. Endocr. Rev. 19:365–396. doi:10.1210/edrv.19.4.0337.

Lu, S. C. 2013. Glutathione synthesis. Biochim. Biophys. Acta 1830:3143–3153. doi:10.1016/j.bbagen.2012.09.008.

MacDonald, H. B. 2000. Conjugated linoleic acid and disease prevention: a review of current knowledge. J. Am. Coll. Nutr. 19:111S–118S.

Markiewski, M. M., and J. D. Lambris. 2007. The role of complement in inflammatory diseases from behind the scenes into the spotlight. Am. J. Pathol. 171:715–727. doi:10.2353/ajpath.2007.070166.

Marnett, L. J. 1999. Lipid peroxidation-DNA damage by malondialdehyde. Mutat. Res.

424:83–95.

Masso-Welch, P. A., D. Zangani, C. Ip, M. M. Vaughan, S. Shoemaker, R. A. Ramirez, and M. M. Ip. 2002. Inhibition of angiogenesis by the cancer chemopreventive agent conjugated linoleic acid. Cancer Res. 62:4383–4389.

Maynard, S., S. H. Schurman, C. Harboe, N. C. de Souza-Pinto, and V. A. Bohr. 2009. Base excision repair of oxidative DNA damage and association with cancer and aging.. Carcinogenesis 30:2–10. doi:10.1093/carcin/bgn250.

Medzhitov, R. 2008. Origin and physiological roles of inflammation. Nature 454:428–435. doi:10.1038/nature07201.

Meller, S. M., E. Stilp, C. N. Walker, and C. Mena-Hurtado. 2013. The link between vasculogenic erectile dysfunction, coronary artery disease, and peripheral artery disease: role of metabolic factors and endovascular therapy.. J. Invasive Cardiol. 25:313–319.

Miller, J. K., E. Brzezinska-Slebodzinska, and F. C. Madsen. 1993. Oxidative stress, antioxidants, and animal function. J. Dairy Sci. 76:2812–2823. doi:10.3168/jds.S0022-0302(93)77620-1.

Mohebi-Nejad, A., and B. Bikdeli. 2014. Omega-3 supplements and cardiovascular diseases.. Tanaffos 13:6–14.

Murakami, M., and T. Hirano. 2012. The molecular mechanisms of chronic inflammation development.. Front. Immunol. 3:323. doi:10.3389/fimmu.2012.00323.

Nagpal, R., H. Yadav, A. K. Puniya, K. Singh, S. Jain, and F. Marotta. 2007. Conjugated linoleic acid: sources, synthesis and potential health benefits - An overview 5:55–65.

Neeley, W. L., and J. M. Essigmann. 2006. Mechanisms of formation, genotoxicity, and mutation of guanine oxidation products. Chem. Res. Toxicol. 19:491–505. doi:10.1021/tx0600043.

Newton, K., and V. M. Dixit. 2012. Signaling in innate immunity and inflammation. Cold Spring Harb. Perspect. Biol. 4:3. doi:10.1101/cshperspect.a006049.

O'Keefe, J. H., and W. S. Harris. 2000. From Inuit to implementation: omega-3 fatty acids come of age. Mayo Clin. Proc. 75:607–614.

Ochoa, J. J., A. J. Farquharson, I. Grant, L. E. Moffat, S. D. Heys, and K. W. J. Wahle. 2004. Conjugated linoleic acids (CLAs) decrease prostate cancer cell proliferation: different molecular mechanisms for cis-9, trans-11 and trans-10, cis-12 isomers.. Carcinogenesis 25:1185–1191. doi:10.1093/carcin/bgh116.

Ostrowska, E., M. Muralitharan, R. F. Cross, D. E. Bauman, and F. R. Dunshea. 1999. Dietary conjugated linoleic acids increase lean tissue and decrease fat deposition in growing pigs.. J. Nutr. 129:2037–2042.

Pannala, V. R., and R. K. Dash. 2015. Mechanistic characterization of the thioredoxin system in the removal of hydrogen peroxide. Free Radic. Biol. Med. 78:42–55. doi:10.1016/j.freeradbiomed.2014.10.508.

Pariza, M. W., S. H. Ashoor, F. S. Chu, and D. B. Lund. 1979. Effects of temperature and time on mutagen formation in pan-fried hamburger.. Cancer Lett. 7:63–69.

Penning, T. M. 2004. Aldo-keto reductases and formation of polycyclic aromatic hydrocarbon o-quinones.. Methods Enzymol. 378:31–67. doi:10.1016/S0076-6879(04)78003-9.

Petit, H. V. 2002. Effects of fatty acids on reproduction in the dairy cow: The Good and the Bad. Proc. Pacific North West Anim. Nutr. Con.

Qin, L., D. Zhao, J. Xu, X. Ren, E. F. Terwilliger, S. Parangi, J. Lawler, H. F. Dvorak, and H. Zeng. 2013. The vascular permeabilizing factors histamine and serotonin induce angiogenesis through TR3/Nur77 and subsequently truncate it through thrombospondin-1.. Blood 121:2154–2164. doi:10.1182/blood-2012-07-443903.

Rainbow, R., W. Ren, and L. Zeng. 2012. Inflammation and Joint Tissue Interactions in OA: Implications for Potential Therapeutic Approaches. Arthritis 2012:1-8. doi:10.1155/2012/741582.

Reiser, R., and H. G. Ramakrishna Reddy. 1956. The hydrogenation of dietary unsaturated fatty acids by the ruminant. J. Am. Oil Chem. Soc. 33:155–156. doi:10.1007/BF02638203.

Ricciotti, E., and G. A. FitzGerald. 2011. Prostaglandins and inflammation.. Arterioscler. Thromb. Vasc. Biol. 31:986–1000. doi:10.1161/ATVBAHA.110.207449.

Robinson, J. G., and N. J. Stone. 2006. Antiatherosclerotic and Antithrombotic Effects of Omega-3 Fatty Acids. Am. J. Cardiol. 98:39–49. doi:10.1016/j.amjcard.2005.12.026

Roca Fernandez, A. I., and A. Gonzalez Rodriguez. 2012. Effect of Dietary and Animal Factors on Milk Fatty Acids Composition of Grazing Dairy Cows: A Review. Iran. J. Appl. Anim. Sci. 2:97–109.

Rodriguez Silva R., Oliveira Rodrigues L. B., de Melo Lisboa M., Mágna Silva Pereira M. and S. Oliveira de Souza. 2014. Conjugated Linoleic Acid (CLA): A Review. I. J. A. S. T. 4 (2):154-170

Rodríguez-Castañedas, J. L., M. J. Peña-Egido, M. García-Marino, and C. García-Moreno. 2011. Quantitative determination of conjugated linoleic acid isomers by silver ion HPLC in ewe milk fat. J. Food Compos. Anal. 24:1004–1008. doi:10.1016/j.jfca.2010.10.009.

Rodríguez-Hernández, H., L. E. Simental-Mendía, G. Rodríguez-Ramírez, and M.A. Reyes-Romero. 2013. Obesity and Inflammation: Epidemiology, Risk Factors, and Markers of Inflammation. Int. J. Endocrinol. 2013:1–11. doi:10.1155/2013/678159.

Rola-Pleszczynski, M., and J. Stankova. 1992. Cytokine gene regulation by PGE(2), LTB(4) and PAF. Mediators Inflamm. 1:5–8. doi:10.1155/S0962935192000024.

Schieber, M., and N. S. Chandel. 2014. ROS Function in Redox Signaling and Oxidative Stress. Curr. Biol. 24:R453–R462. doi:10.1016/j.cub.2014.03.034.

Schönfeld, P., and L. Wojtczak. 2008. Fatty acids as modulators of the cellular production of reactive oxygen species. Free Radic. Biol. Med. 45:231–241. doi:10.1016/j.freeradbiomed.2008.04.029.

Schreibelt, G., J. van Horssen, S. van Rossum, C. D. Dijkstra, B. Drukarch, and H. E. de Vries. 2007. Therapeutic potential and biological role of endogenous antioxidant enzymes in multiple sclerosis pathology. Brain Res. Rev. 56:322–330. doi:10.1016/j.brainresrev.2007.07.005.

Shearer, G. C., O. V Savinova, and W. S. Harris. 2012. Fish oil -- how does it reduce plasma triglycerides?. Biochim. Biophys. Acta 1821:843–851. doi:10.1016/j.bbalip.2011.10.011.

SHORLAND, F. B., R. O. WEENINK, and A. T. JOHNS. 1955. Effect of the rumen on

dietary fat.. Nature 175:1129–1130.

Shultz, T. D., B. P. Chew, and W. R. Seaman. Differential stimulatory and inhibitory responses of human MCF-7 breast cancer cells to linoleic acid and conjugated linoleic acid in culture.. Anticancer Res. 12:2143–2145.

Shultz, T. D., B. P. Chew, W.R. Seaman, and L. O. Luedecke. 1992. Inhibitory effect of conjugated dienoic derivatives of linoleic acid and beta-carotene on the in vitro growth of human cancer cells.. Cancer Lett. 63:125–133.

Simopoulos, A. 2016. An Increase in the Omega-6/Omega-3 Fatty Acid Ratio Increases the Risk for Obesity. Nutrients 8:128. doi:10.3390/nu8030128.

Simopoulos, A. P. 2008. Omega–6/Omega–3 Essential Fatty Acids: Biological Effects. KARGER, Basel.

Simopoulos, A. P. 2002. Omega-3 fatty acids in inflammation and autoimmune diseases.. J. Am. Coll. Nutr. 21:495–505.

Singh, S., C. Brocker, V. Koppaka, Y. Chen, B. C. Jackson, A. Matsumoto, D. C. Thompson, and V. Vasiliou. 2013. Aldehyde dehydrogenases in cellular responses to oxidative/electrophilicstress. Free Radic. Biol. Med. 56:89–101. doi:10.1016/j.freeradbiomed.2012.11.010.

Sordillo, L. M., and W. Raphael. 2013. Significance of Metabolic Stress, Lipid Mobilization, and Inflammation on Transition Cow Disorders. Vet. Clin. North Am. Food Anim. Pract. 29:267–278. doi:10.1016/j.cvfa.2013.03.002.

Sordillo, L. M., G. A. Contreras, and S.L. Aitken. 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Anim. Heal. Res. Rev. 10:53–63. doi:10.1017/S1466252309990016.

Steinhart, H., R. Rickert, and K. Winkler. 2003. Identification and analysis of conjugated linoleic acid isomers (CLA).. Eur. J. Med. Res. 8:370–372.

Tian, R., G. Hou, D. Li, and T.-F. Yuan. 2014. A possible change process of inflammatory cytokines in the prolonged chronic stress and its ultimate implications for health. ScientificWorldJournal. 2014:1-8. doi:10.1155/2014/780616.

Tilley, S. L., Coffman T. M., and B. H. Koller. 2001. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes.. J. Clin. Invest. 108:15–23. doi:10.1172/JCI13416.

Tretyakova, N. Y., A. Groehler, S. Ji, and S. Ji. 2015. DNA-Protein Cross-Links: Formation, Structural Identities, and Biological Outcomes.. Acc. Chem. Res. 48:1631–44. doi:10.1021/acs.accounts.5b00056.

Trevisi, E., A. Zecconi, G. Bertoni, and R. Piccinini. 2010. Blood and milk immune and inflammatory profiles in periparturient dairy cows showing a different liver activity index. J. Dairy Res. 77:310–317. doi:10.1017/S0022029910000178.

Turner, M. D., B. Nedjai, T. Hurst, and D. J. Pennington. 2014. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. Biochim. Biophys. Acta - Mol. Cell Res. 1843:2563–2582. doi:10.1016/j.bbamcr.2014.05.014.

Tüzün, A., A. Erdil, V. Inal, A. Aydin, S. Bağci, Z. Yeşilova, A. Sayal, N. Karaeren, and K. Dağalp. 2002. Oxidative stress and antioxidant capacity in patients with inflammatory bowel disease.. Clin. Biochem. 35:569–572.

Vaughan, V. C., P. Martin, and P. A. Lewandowski. 2013. Cancer cachexia: impact, mechanisms and emerging treatments. J. Cachexia. Sarcopenia Muscle 4:95–109. doi:10.1007/s13539-012-0087-1.

Wall, R., R. P. Ross, G. F. Fitzgerald and C. Stanton. 2010. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr. Rev. 68:280–289. doi:10.1111/j.1753-4887.2010.00287.x.

Wardhana, E. S. Surachmanto, and E. A. Datau. 2011. The role of omega-3 fatty acids contained in olive oil on chronic inflammation.. Acta Med. Indones. 43:138–143.

West, D. B., J. P. Delany, P. M. Camet, F. Blohm, A. A. Truett, and J. Scimeca. 1998. Effects of conjugated linoleic acid on body fat and energy metabolism in the mouse. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 275: R667-R672

2. CHAPTER 1

Conjugated Linoleic Acid (CLA) Isomers Strongly Improve the Redox Status of Bovine Mammary Epithelial Cells (BME-UV1)

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2.1 Abstract

Some studies have shown the protective effects of CLA isomers against oxidative stress and lipid peroxidation in animal models but no information is available about CLA and changes in oxidative status of bovine mammary gland. The objectives of the study were to assess in vitro the effect of CLA on the cellular antioxidant response of bovine mammary cells, to examine whether CLA isomers could play a role in cell protection against the oxidative stress and to study the molecular mechanism involved. For the study BME-UV1cells, a bovine mammary epithelial cell line, were used as experimental model. BME-UV1 cells were treated with complete medium containing 50 µM of cis-9,trans-11 CLA (c9,t11 CLA), trans-10,cis-12 CLA (t10,c12 CLA) and CLA Mixture (1:1, c9,t11: t10,c12 CLA). To monitor cellular uptake of CLA isomers, cells and culture medium were collected at 0, 3 and 48 h from CLA addition for lipid extraction and fatty acids analyses. To assess the cellular antioxidant response, glutathione (GSH/GSSH), nicotinamide adenine dinucleotide phosphate (NADPH), and γ-Glutamyl-cysteine ligase (γGCL) activity were measured after 48 hours from addition of CLA. Cytoplasmic superoxide dismutase (SOD), glutathione peroxidase (GPx1), glutathione S-transferase (GST) and glutathione reductase (GR) activities and mRNA were also determined. Intracellular Reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS) production were assessed in cells supplemented with CLA isomers. Cell viability after 3 h to H2O2 exposure was assessed in order to evaluate and to compare the potential protection of different CLA isomers against H2O2-induced oxidative stress. Mammary cells readily picked up all CLA isomers, their accumulation was time dependent and main metabolites at 48 hours are two 18:3 isomers. CLA treatment induces an intracellular GSH increase, match by high concentration of NADPH and an increase of γGCL activity, mainly in cells treated with t10,c12 CLA isomer. CLA isomers treatment of bovine mammary cells increases SOD, GPx1, GST activity and decrease GR activity, but no changes in gene expression of these antioxidant enzymes were observed. Cells supplemented with CLA isomers show a reduction in intracellular ROS and TBARS levels. All CLA isomers were able to enhance cell resistance against H2O2-induced oxidative stress. These suggest an antioxidant role of CLA, in particular of t10,c12 CLA, by developing a significantly high redox status in cells.

Keywords: cis-9,trans-11 CLA, trans-10,cis-12 CLA, bovine mammary gland cells, oxidative stress.

2.2 Introduction

In the dairy cows, the transition period is particularly important for health and subsequent performance of these animals, which are exposed to drastic physiological changes and metabolic stress (Grummer, 1993; Goff and Horst, 1997; Drackley, 1999). Usually, cows in this transition phase are secreting more energy than they consume, as a consequence, animals experience a substantial negative energy balance (**NEBAL**) and often even oxidative stress (Bernabucci et. al, 2005). Substantial evidence corroborates that oxidative stress during

the pre-partum and early lactation period may contribute to a number of health disorders in dairy cattle (Bernabucci et al., 2005; Sordillo et al., 2009). A management method utilized in dairy cows to alleviate the magnitude of NEBAL was feeding rumen-protected conjugated linoleic acid (CLA). The influence of CLA on milk composition has been evaluated in a number of studies and it has been shown that *trans-10,cis-12* CLA treatment results in milk fat depression, whereas *cis-9,trans-11* CLA was not effective in milk fat depression (Baumgard et al., 2000; Bauman et al., 2008). Some authors (Baumgard et al., 2000a, 2000b; Mackle et al., 2003) reported that *trans*-10,*cis*-12 have little or no effect on basal plasma concentrations of metabolites (glucose, non-esterified fatty acids, β-hydroxybutyrate) and hormones (leptin, insulin, IGF-I) associated with energy homeostasis. In contrast, other studies demonstrated a significant effect of CLA supplementation (as a mix of *trans*-10,*cis*-12 and *cis-9,trans-11* CLA) on circulating IGF-1 and adiponectin in transition dairy cows, resulting in a prolongation of peripartal insulin resistance and drain of nutrients towards the mammary gland (Castañeda-Gutiérrez et al., 2005; Singh et al., 2014).

The effects of *trans*-10,*cis*-12 CLA in dairy cows appear to be specific to the mammary gland. Moreover, *trans*-10,*cis*-12 CLA effects may depend on the dietary status and the stage of lactation of the studied cows (Bauman and Griinari, 2003; Selberg et al., 2004; Castañeda-Gutiérrez et al., 2005; Odens et al., 2007). Hötger et al. (2013) reported that CLA supplementation (as a mix of *trans*-10,*cis*-12 and *cis-9,trans-11* CLA) in dairy cows reduces endogenous glucose production during early lactation, most likely due to lower glucose utilization for milk fat synthesis and a more efficient whole-body energy utilization in CLA-fed cows. Von Soosten et al. (2012) as well as Pappritz et al. (2011) concluded that *trans*-10,*cis*-12 CLA-fed cows have an increased efficiency of metabolizable energy utilization.

Given that the administration of *trans*-10,*cis*-12 CLA in mammary cells induces a reduction in the synthesis of milk fat, and this may leads to an increased availability of glucose, we hypothesized that glucose sparing might be used for cellular activities related to oxidative response other than lactose and milk synthesis. To date, there is no information available concerning the relationship between administration of CLA isomers and changes in oxidative status of bovine mammary gland. Some studies have shown the protective effects of CLA isomers (mainly *cis-9,trans-11* CLA) against oxidative stress and lipid peroxidation in animal models (Andreoli et al., 2010; Chinnadurai et al., 2013). On the other hand, adverse effects of *trans*-10,*cis*-12 CLA regarding increased susceptibility to lipid autoxidation and

enhancing fatty acid oxidation have also been reported in 3T3-L1 cells and in animal models (Da Silva Marineli et al., 2012; den Hartigh et al., 2013). However, mechanism of action is not well understood, yet. Therefore, the study aims to assess in vitro effects of *trans*-10,*cis*-12 and *cis-9,trans-11* CLA isomers on the cellular antioxidant response of bovine mammary cells, to examine whether these CLA isomers could play a role in cell protection against the oxidative stress and to study the molecular mechanism involved.

2.3 Materials and Methods

2.3.1 Cell Culture Conditions

The BME-UV1 cell line was created at the University of Vermont from primary bovine mammary epithelial cells in culture by stable transfection with SV-40 large T-antigen (Zavizion et al. 1996). BME-UV1 cells were kindly provided by Dr. Antonella Baldi, Department FENS, University of Milan, Italy. Cells were routinely cultivated into 75 cm^2 tissue culture flasks (Costar, Corning, NY, USA), in a mixture of 50% DMEM-F12, 30% RPMI-1640 and 20% NCTC-135 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS), 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 1 µg/ml insulin, 5 µg/ml transferrin, 1 µg/ml hydrocortisone, 0.5 µg/ml progesterone, 10 µg/ml L-ascorbic acid and antibiotics (penicillin 100 IU/ml; streptomycin 100 µg/ml). All medium supplements were from Sigma-Aldrich. The cells were maintained at 37°C in a humidified 5% CO_2 incubator until confluence. Cells used in the present work were at passage numbers between 39 and 41.

2.3.2 Experimental Design

In order to examine the role of CLA on oxidative status of bovine mammary gland, BME-UV1 cells were re-suspended in complete culture medium to a concentration of 5×10^5 cells/ml and dispensed into wells of a 6-well and 96-well tissue culture plates. After 24 h of incubation at 37°C in an atmosphere of 5% CO_2, the medium was removed and cells were treated with complete medium containing 50 µM of *cis-9,trans-11* CLA (c9,t11 CLA), *trans-10,cis-12* CLA (t10,c12 CLA) and CLA Mixture (50% c9,t11 and 50% t10,c12 CLA). In order to monitor cellular uptake of CLA isomers, cells were treated as described above and samples

of cells and culture medium were collected at 0, 3 and 48 h from CLA addition for lipid extraction and FA analyses.

To study the cellular antioxidant response, the role of CLA in cell protection against the oxidative stress and the molecular mechanism involved, cell samples were collected for cell extracts and mRNA extraction after 48 hours from addition of CLA. To test the potential protection of CLA against hydrogen peroxide (H_2O_2)-induced oxidative stress, cells were treated as described above followed by incubation at 37°C for 3 h with H_2O_2 (50 and 100 µM) and cell viability was determined. CLA concentration (50 µM) was used because at 50 µM concentration no differences were observed in cell viability between CLA treatments and control. Moreover previous studies have shown that higher concentrations of CLA induce apoptosis in the mammary cells (Keating et al., 2007; Wang et al., 2014). Finally, physiological concentration of CLA in human sera was in the range of 10-70 µM (De La Torre et al., 2005). Therefore, the concentration of 50 µM used in the present study is close to physiological levels. CLA isomers were purchased from Larodan (Malmo, Sweden).

The experiments included at least three replicates per treatment and were repeated at least twice.

2.3.3 CLA Uptake by Cells

Lipid extraction. Total lipids of cell pellet and culture medium were extracted according to a modified Folch (1957) procedure. Briefly, the whole cell pellet (nearly one million of cells) or 0.1 mL of culture medium were dissolved in a screw cap tube with 12 mL of chloroform/methanol (2/1 vol/vol) added with 2 µg of vitamin E and left in the dark at room temperature for 60 min. Then 4 mL of water were added and tubes were centrifuged at 900 *g* for 60 min in order to obtain two phases. After centrifugation the lower phase containing chloroform and total lipids was collected. The extracted fat was dried at 30°C by means of a rotary evaporator device.

Preparative Procedure for HPLC analysis. Preparation of free FAs was obtained by mild saponification according to Melis et al. (2001): lipid extracts were dissolved in 5 ml of ethanol, 0.1 mL of desferal in water (25 mg/ml), 1 mL of a 25% water solution of ascorbic acid, 0.5 ml of 10N KOH, left in the dark at room temperature for 14 h. Then 10 mL of n-hexane and 7 mL of water were added and the solution was acidified by adding 37% HCl, to a pH 3-4. Samples were vortexed for 1 min., then, centrifuged at 900 *g* for 60 min. Finally,

solvent was evaporated and the residue was dissolved in 0.5 mL of a mixture acetic acid 0.14% (vol/vol) in acetonitrile.

DAD HPLC analysis of free fatty acids. Analysis of free fatty acids was carried out with a Varian Dual Prostar 210 liquid chromatograph (Varian, Palo Alto, CA, USA) equipped with a diode array detector. A OmniSpher 5 C18 column, 5 μm particle size (Chrompack, Middelburg, The Netherlands), 250x4.6 mm was used with a mobile phase of CH_3CN/ H_2O/CH_3COOH (70/30/0.12, v/v/v) and a flow rate of 1.5 ml/min. Unsaturated non-conjugated fatty acids were detected at 200 nm, conjugated diene fatty acids at 234 nm. CLA isomers were recognized by using a commercial standard mixture of c9,t11 CLA and t10,c12 CLA and pure c9,t11 CLA or t10,c12 CLA standards (Matreya Inc., Pleasant Gap, PA, USA). Since CLA metabolites are not commercially available, their recognition was obtained by diode array spectra and by comparison with published HPLC profiles (Melis et al., 2001).

2.3.4 Cellular Antioxidant Response and Cell Protection

Thiol redox status and antioxidant enzymes. The thiol redox status of BME-UV1, cultured under the conditions described above, was assessed by measuring the content of glutathione (GSH/GSSH), the content of nicotinamide adenine dinucleotide phosphate (NADPH), and the activity of γ-Glutamyl-cysteine ligase (γGCL).

Cytoplasmic superoxide dismutase (SOD), glutathione peroxidase (GPx1), glutathione S-transferase (GST) and glutathione reductase (GR) activities were determined in BME-UV1 cultured under the conditions described above and were measured using colorimetric assays.

For the determination of GSH/GSSG and NADPH adherent cells were detached using trypsin/EDTA solution and centrifuged at 4,500 x g for 5 min. The pellet was re-suspended in 200 μL of PBS and lysed by sonication at 100 W for 60 sec, centrifuged (15,000 x g for 5 min at 4°C) and stored at -20°C until analysis. In cell extracts NADPH and GSH/GSSH concentrations were determined by colorimetric assays (BioAssay Systems, Hayward, CA, USA). Optical density (**OD**) was measured by a spectrophotometer at 405 and 540, respectively. γGCL activity was determined by a fluorescence assay as described by Chen et al (2010). BME-UV1 cells were detached using trypsin/EDTA solution and centrifuged at 4,500 x g for 5 min. The pellet was re-suspended in 100 μL of TES/SB buffer (w/v, 1/4) consisting of 20 mM Tris, 1 mM EDTA, 250 mM sucrose, 20 mM sodium borate and 2 mM

serine. The cells were sonicated at 100 W for 60 sec and then centrifuged at 10,000 x g at 4˚C for 10 min. The supernatants were collected and centrifuged again at 15,000 x g at 4˚C for 20 min. The supernatants were collected and the protein concentrations were determined using a BCA Protein Assay Kit from Pierce (Rockford, IL, USA), with BSA used as the standard. For the γGCL activity assay, aliquots of 30 µl supernatant were mixed with 30 µl γGCL reaction cocktail (400 mM Tris, 40 mM ATP, 40 mM L-glutamic acid, 2 mM EDTA, 20 mM sodium borate, 2 mM serine and 40 mM MgCl2). Following incubation at 37˚C for 5 min, 30 µl cysteine solution (30 mM; dissolved in TES/SB buffer) was added and the mixtures were incubated at 37˚C for 13 min. The enzymatic reaction in the mixture, was stopped by precipitating proteins with 200 mM 5-sulfosalicylic acid (**SSA**). After placing on ice for 20 min, the mixtures were centrifuged at 2,000 x g at 4˚C for 10 min. Following centrifugation, 20µl of each supernatant containing γ-glutamylcysteine (**γGC**) were added to a 96 well plate designed for fluorescence detection. For each assay, 20 µl γGC standards, containing 5 µl γGCL reaction cocktail [5 µl SSA (200 mM), 5 µl H2O and 5 µl γGC standard solution (0, 20, 40, 60, 80, 100, 120 and 140 µM in TES/SB buffer)], was added to each well of the same 96 well plate to generate a standard curve. Next, 180 µl 2,3-naphthalenedicarboxyaldehyde (**NDA**) was added to each well. Following incubation in the dark at room temperature for 30 min, the formation of NDA-γGC was measured (472 nm excitation/528 nm emission) using a fluorescent plate reader (Multimode Detector DTX 880, Beckman Coulter, Inc; Austria). The production of γGC in each sample was calculated using the standard curve. Values were expressed in µM per µg of protein. To determine GPx1, GST, GR and SOD activity, after incubation, the plates were placed in ice, the monolayers were mechanically detached with a scraper and the cells were sonicated (100 W for 60 sec), centrifuged (15,000 x g for 5 min at 4°C) and stored at -80°C until analysis. The enzymatic activities were determined by colorimetric assays (BioAssay Systems, Hayward, CA, USA). Optical density (OD) was measured by a spectrophotometer at 340, 405 and 450 nm, respectively.

RNA isolation and real-time PCR. mRNA abundance of bovine antioxidant enzymes (GPx1, GSR, GST and SOD) were assayed in BME-UV1 cultured under the conditions described above and were carried out by real time PCR. In order to isolate total RNA, BME-UV1 cells were seeded in 6-well tissue culture plate at the concentration of 5×10^5 cells/ml in complete medium containing 10% FBS. Total RNA was extracted by QIAzol Lysis Reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions and stored at -80°C.

RNA was quantified using Quant-iT RNA assay Kit (Invitrogen, Carlsbad, CA, USA) and fluorescence was measured at excitation/emission of 644/673 nm. The integrity of the RNA was checked by visualization of 18S and 28S ribosomal bands on an agarose gel. One microgram of total RNA was reverse transcribed using Quantitect reverse transcription kit (Qiagen) in a total volume of 20 µl on a PCR Express thermal cycler (Hybaid, Ashford, UK). Quantitative SYBR Green real-time PCR was performed following the manufacturer's recommendations using LightCycler (Roche). To account for possible variation related to cDNA input or the presence of PCR inhibitors, the endogenous reference gene RPS9 was simultaneously quantified for each sample, and data were normalized accordingly. In Table 1 the specific characteristics of primers used for the real-time PCR are shown. PCR products were subjected to a melting curve analysis on the LyghtCycler and subsequently 2% agarose/Tris–borate–EDTA gel electrophoresis to confirm amplification specificity and amplicon size. To allow relative quantification after PCR, standard curves were constructed from the standard reactions for each target and housekeeping genes by plotting crossing point **(Cp**) values, i.e., the cycle number at which the fluorescence signal exceeds background versus log cDNA dilution. The Cp readings for each of the unknown samples where then used to calculate the amount of either the target or housekeeping relative to the standard, using the second derivative maximum method with the LyghtCycler analysis software 3.5 (Roche Applied Science).

Reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS) production. To determine ROS concentration, cells were washed twice with PBS and incubated with 20 µM 2',7'-Dichlorodihydrofluorescin diacetate (**DCFH-DA**) probe in PBS at 37°C for 40 min. Fluorescence was measured at 485 nm (excitation) and 535 nm (emission) wavelengths on a microplate reader (Multimode Detector DTX 880, Beckman Coulter, Inc; Austria). For the determination of TBARS, adherent cells were detached using trypsin/EDTA solution

Table 1. DNA sequences of bovine sense and antisense primers used for real time PCR analysis.

Gene	Primer	Temperature of annealing, °C	Sequence
GPx1 forward	CTTCCCCTGCAAC CAGTTTG	60	NM_174076
GPx1 reverse	GGCAATTCAGGA TCTCCTCGTT		
GR forward	GACCAAACCATC GAAGGAGAGTAT	60	NM_001114190
GR reverse	CTCGTCCAAGCAT CTCTTCCT		
CuZn SOD forward	GAAGAGAGGCAT GTTGGAGA	57	NM_174615
CuZn SOD reverse	CCAATTACACCAC GAGCCAA		
GST1 forward	AATGGACGTGGC AGAATGGAGT	60	NM_177515
GST1 reverse	GGGCACAGTGGT AAATGCATGA		
RPS9 forward	GGCGGCTCGTCC GTATC	60	XN_864261
RPS9 reverse	AATCTTCAGGCCC AGGATGTAATC		

and centrifuged at 4,500 x g for 5 min. The pellet was re-suspended in 200 µL of PBS and lysed by two cycles of sonication at 100 W for 30 sec, centrifuged (15,000 x g for 5 min at 4°C) and stored at -20°C until analysis. In cell extracts TBARS concentrations were determined by colorimetric assays (BioAssay Systems, Hayward, CA, USA). Optical density (OD) was measured by a spectrophotometer at 340 nm.

Cells viability. BME-UV1 cells were seeded into 96-well microplates at an optimal density (5×10^5 cells/ml) and were grown with different CLA isomers (c9,t11 CLA, t10,c12 CLA and CLA Mixture) for 48 h. Then cells were incubated at 37°C for 3 h with different concentrations (50 and 100 µM) of H_2O_2. Cell viability before and after incubation with H_2O_2 was determined using XTT assay with Cell Proliferation kit II [XTT: sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; Roche Applied Science, Indianapolis, IL, USA] according to the manufacturer's instructions. For each treatment, after 3 h of exposure to H_2O_2, 50 µl of XTT labeling mixture was added to each well. After 4 h incubation at 37°C, absorbance was measured at 450 nm. Background absorbance was subtracted from each value. Results were expressed as optical density (OD450).

2.3.5 Statistical analysis

All data of the experiment are presented as LSmeans and standard error of the means (**SEM**). The data were analyzed by ANOVA using Statistica-7 Software package (Stat Soft, Inc., USA). The significance of the differences was assessed by the Fisher's Least Square Difference (LSD) test. Significance was declared at $P < 0.05$.

2.4 Results

2.4.1 Incorporation of CLA Isomers in Cell Lipids

The cellular uptake of fatty acids was determined at 3 and 48 h incubation in the presence of c9,t11 CLA, t10,c12 CLA and CLA Mix. The disappearance of the fatty acids from the medium and the appearance in the cells was used as a measure of fatty acids uptake. As shown in Table 2 the average cellular uptake of fatty acids during the 3 h incubation was 23%, 22.85% and 19.52% for c9,t11 CLA, t10,c12 CLA and CLA mix, respectively. After 48 h fatty acids uptake rose to 73.25%, 71.88% and 78.60% for c9,t11 CLA, t10,c12 CLA and CLA mix, respectively. This is also confirmed by a simultaneous decrease of CLA isomers from the culture medium. All fatty acids were highly incorporated. Cells cultured with c9,t11 CLA led to the highest cellular contents of total fatty acids. During the 3 h and 48 h incubation a progressive enrichment of C18:3 c6c9t11 and C18:3 c6t10c12 as CLA metabolites was observed within cells. No significance variation was noted concerning the differences between each fatty acid for uptake rates.

Table 2. Concentration of fatty acids in BME-UV1 cells and culture medium after 3 h and 48 h conjugated linoleic acid (CLA) isomers incubation. Cells and culture medium before incubation (time = 0) were used as control. Data are expressed as mean ± SEM (n = 6).

	Cells (nmol/10^6 cells)			Culture medium (nmol/mL)		
Fatty acids	Control	3 h	48 h	Control	3 h	48 h
C18:2 c9,t11						
C18:2 c9,t11	0.36 ± 0.07	1.92 ± 0.76	9.11 ± 0.54	0.25 ± 0.0004	5.75 ± 0.59	3.65 ± 0.64
C18:3 c6,c9,t11	nd	0.16 ± 0.06	1.24 ± 0.08	nd	nd	nd
C:18:2 t10,c12						
C18:2 t10,c12	0.19 ± 0.03	2.44 ± 0.42	7.93 ± 0.67	0.03 ± 0.0004	8.21 ± 1.03	3.03 ± 0.19
C18:3 c6,t10,c12	nd	0.18 ± 0.04	1.35 ± 0.09	nd	nd	nd
50% C18:2 c9t11 + 50% C:18:2 t10c12 (CLA Mix)						
C18:2 c9,t11	0.36 ± 0.07	0.89 ± 0.22	5.47 ± 0.57	0.25 ± 0.0004	8.56 ± 0.07	1.21 ± 0.03
C18:2 t10,c12	0.19 ± 0.03	0.73 ± 0.16	2.10 ± 0.16	0.03 ± 0.0004	3.40 ± 0.39	0.50 ± 0.03
C18:3 c6,c9,t11	nd	0.05 ± 0.01	0.46 ± 0.06	nd	nd	nd
C18:3 c6,t10,c12	nd	0.08 ± 0.01	0.68 ± 0.07	nd	nd	nd

nd = non detectable

2.4.2 Thiol Redox Status

Oxidized and reduced glutathione. The levels of non-enzymatic antioxidant like GSH are depicted in Figure 1. Quantitative determination of reduced and oxidized GSH (Figure 1A and B, respectively) showed an increase ($P < 0.01$) of reduced GSH in cells treated with t10,c12 CLA, c9,t11 CLA and CLA Mix compared with control; in particular, t10,c12 CLA showed an increase ($P < 0.01$) of reduced GSH compared with c9,t11 CLA and CLA Mix. No differences in the level of oxidized GSH were noted for cells treated with CLA isomers compared with control culture.

Quantitative determination of nicotinamide adenine dinucleotide phosphate (NADPH). Concentrations of a redox coenzyme NADPH are reported in figure 1C. Cells treated with t10,c12 CLA showed higher levels ($P < 0.01$) of NADPH compared with other CLA treatments and control.

γ-Glutamyl-cysteine ligase activity. Thiol redox status was assessed by measuring γGCL activity (Figure 1D). After the 48 h, CLA isomers induced a strong antioxidant response by increasing ($P < 0.01$) intracellular γGCL activity compared with control. No differences of γGCL activity were observed for different CLA treatments.

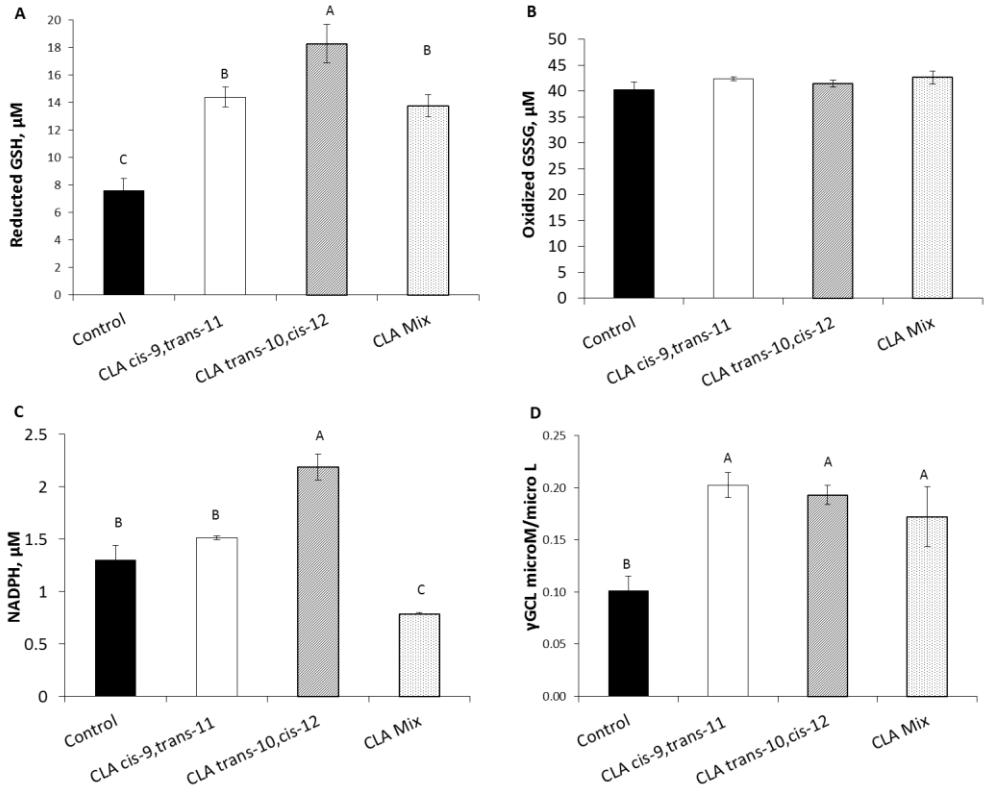

Figure 1. Intracellular concentrations of reduced (GSH; A) and oxidized (GSSG; B) glutathione, NADPH (C), and γ-glutamate cysteine ligase (γGCL; D) activity in BME-UV1 cells after 48 h of CLA isomer exposure. Data are reported as least squares means ± standard error of the mean (n = 6). Significant differences between the control group and treatments and among treatments are represented by different letters ($^{A,B}P < 0.01$). CLA mix = 50% *cis*-9,*trans*-11/50% *trans*-10,*cis*-12.

Quantification of Activity and mRNA Expression Levels of the Antioxidant Enzymes

The enzymatic activities of GPx1, GR, GST and SOD in cell lysates are shown in Figure 2 (A, B, C and D, respectively). From this figure, it is evident that the activity of GPx1 showed an increase ($P < 0.01$) in cells treated with CLA compared with control. GPx1 activity was greater ($P < 0.01$) in c9,t11 CLA cells in respect to t10,c12 CLA and CLA Mix.

The GR activity decreased ($P < 0.01$) in cells treated with t10,c12 CLA and CLA Mix compared with control cells. In cells treated with c9,t11 CLA, the GR activity was not different compared to control but showed a decrease ($P < 0.05$) compared with other CLA treatments (t10,c12 and Mix). GST activity showed an increase ($P < 0.05$) in cells treated with t10,c12 CLA compared with control. The activity of SOD was increased ($P < 0.01$) by CLA when compared with control. In figure 3A, B, C and D the mRNA expression of GPx1, GR, GST and SOD, respectively is reported. The levels of gene expression of GPx1, GR, GST and SOD mRNA did not show any differences between CLA treatments and control. No

differences were observed among treatments with different CLA isomers (c9,t11, t10,c12 and Mix).

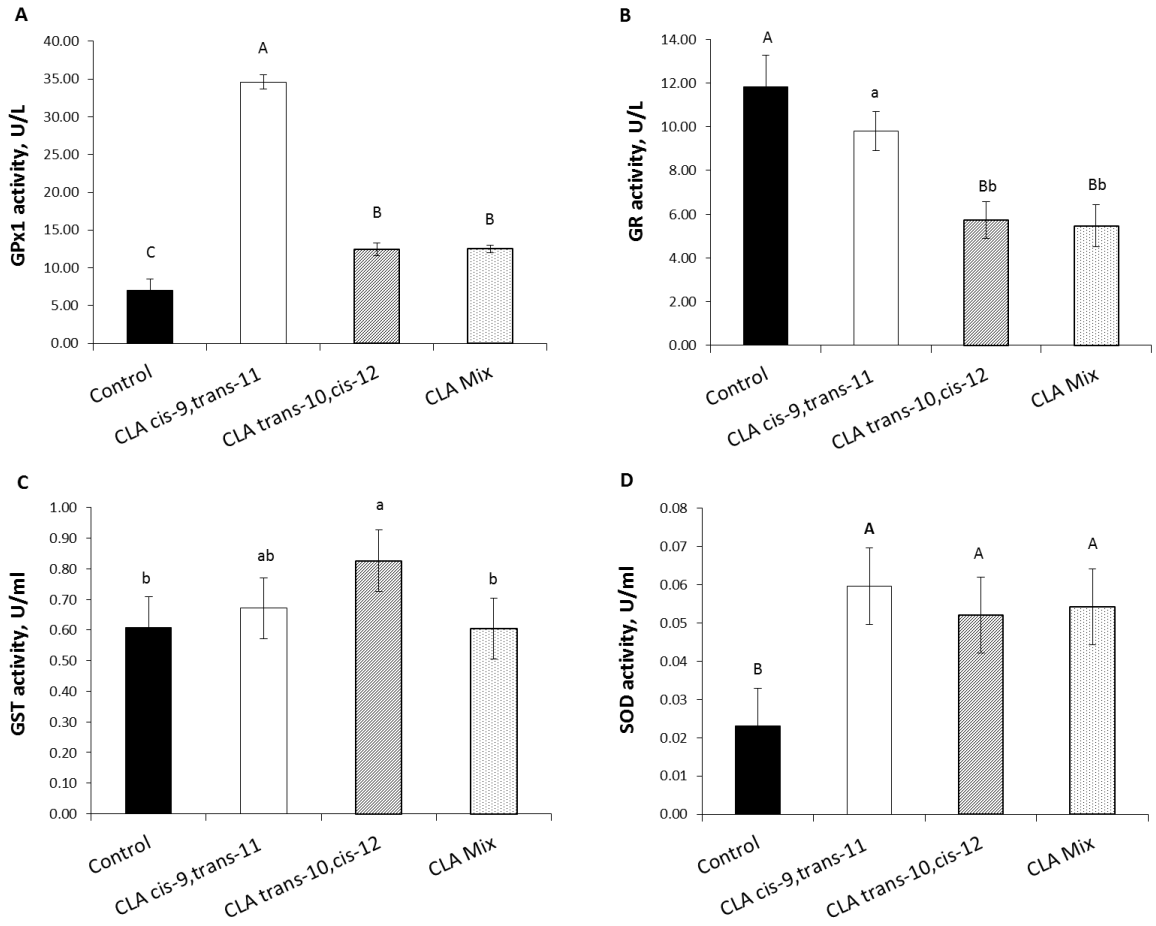

Figure 2. Intracellular levels of glutathione peroxidase (GPx1; A), glutathione reductase (GR) (B), glutathione-S-transferase (GST; C), and superoxide dismutase (SOD; D) activity in BME-UV1 cells after 48 h of CLA isomer exposure. Data are reported as least squares means ± SEM (n = 6). Significant differences between the control group and treatments and among treatments are represented by different letters ($^{A,B}P < 0.01$; $^{a,b}P < 0.05$). CLA mix = 50% *cis*-9,*trans*-11/50% *trans*-10,*cis*-12.

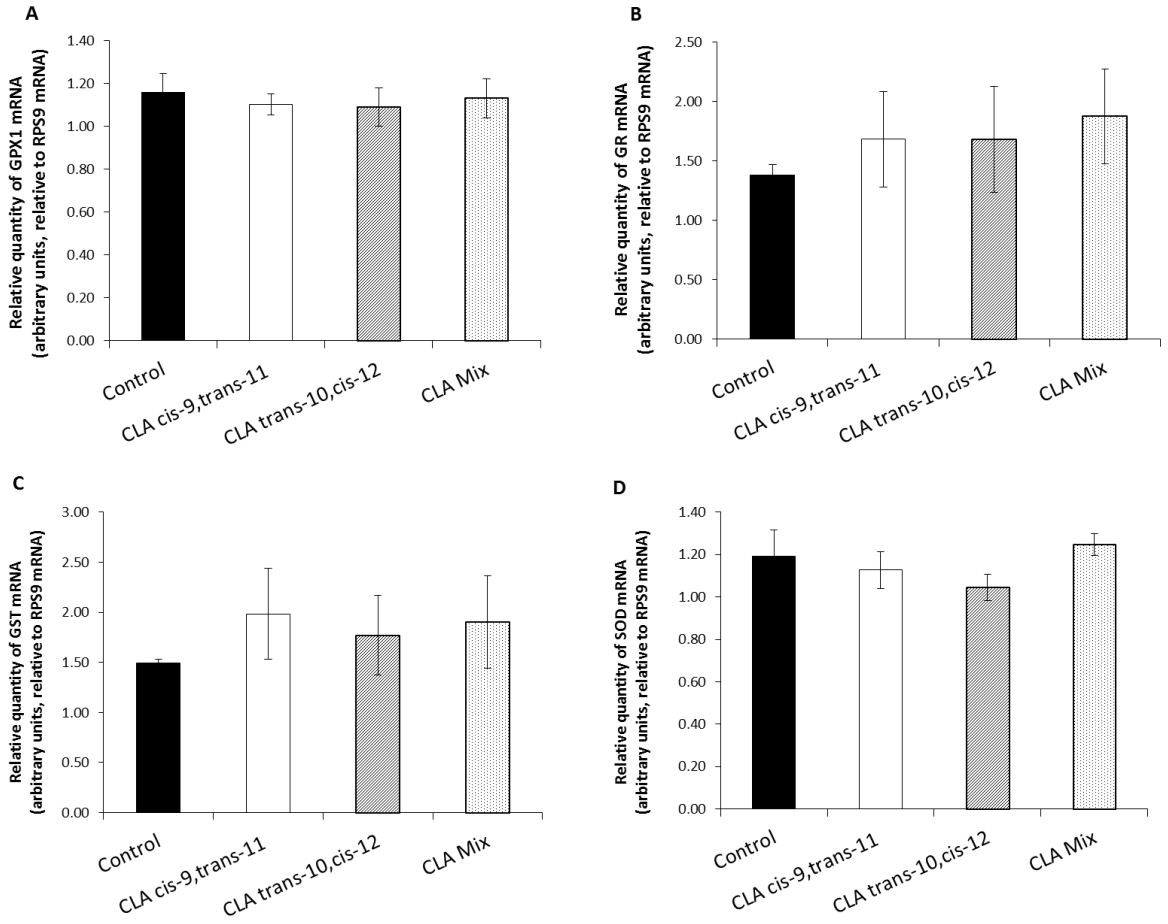

Figure 3. Messenger RNA abundance of glutathione peroxidase (GPx1; A), glutathione reductase (GR; B), glutathione-S-transferase (GST;C), and superoxide dismutase (SOD; D) in BME-UV1 cells after 48 h of CLA isomer exposure. Data are reported as least squares means ± SEM (n = 6). CLA mix = 50% *cis*-9,*trans*-11/50% *trans*-10-*cis*-12.

2.4.3 Intracellular Reactive Oxygen Species (ROS) and Thiobarbituric Acid Reactive Substances (TBARS)

GSH is recognised as the main intracellular antioxidant and it maintains the redox status of the cell, like so, it is related to intracellular ROS levels. For this reason, intracellular ROS production was assessed in cells supplemented with CLA isomers. At 48 h (Figure 4A), ROS showed a 8.6%, 10.6% and 19% reduction in cells treated with c9,t11 CLA ($P < 0.05$), t10,c12 CLA ($P < 0.05$) and CLA mix ($P < 0.01$), respectively, when compared with control. The intracellular ROS levels in cells treated with CLA mix showed a decrease ($P < 0.05$) compared to the other two CLA treatments.

Cell concentrations of TBARS are shown in figure 4B. All CLA treatments reduced ($P < 0.01$) TBARS levels compared with untreated cells.

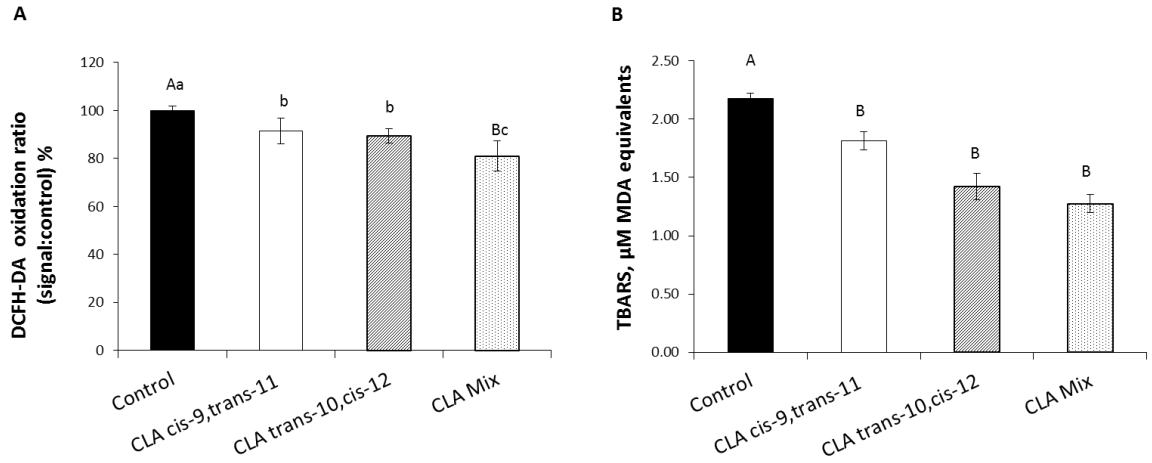

Figure 4. Intracellular production of reacting oxygen species (ROS; A) and thiobarbituric acid reactive substances (TBARS; B) level in BME-UV1 cells after 48 h of conjugated linoleic acid (CLA) isomer exposure. Data are reported as least squares means ± SEM (n = 6). Significant differences between the control group and treatments and among treatments are represented by different letters ($^{A,B}P < 0.01$; $^{a,b}P < 0.05$). CLA mix = 50% *cis*-9,*trans*-11/50% *trans*-10,*cis*-12.

2.4.4 Cells Protection Against Oxidative Stress

Cell viability was carried out using the XTT assay and was evaluated after 48 h of CLA exposure. As showed in table 3 no CLA treatments reduced the cell viability, no differences were observed between treatments with different CLA isomers (c9,t11 CLA, t10,c12 CLA and CLA Mix).

In order to evaluate and to compare the potential protection of different CLA isomers against H_2O_2-induced oxidative stress, we assessed cell viability by the XTT test after 3 h to H_2O_2 exposure. As shown in Table 3, all CLA isomers were able to enhance cell resistance against oxidative stress at 48 h. Cell resistance with c9,t11 CLA, t10,c12 CLA and CLA Mix increased to 122%, 114% and 126%, respectively, compared with the control.

Table 3. Cell viability of BME-UV1 cells (n = 6) observed, respectively, after 48 h of CLA isomer supplementation, and after 3 h of H_2O_2 treatment (50 and 100 μ*M*)[1]

			50 μM H_2O_2 (3h)		100 μM H_2O_2 (3h)	
Supplementation	LSM	SEM	LSM	SEM	LSM	SEM
Control	1,55	0,14	1,32	0,07	1,27	0,01
CLA cis-9,trans-11	1,71	0,06	1.61 **	0,12	1.45 **	0,10
CLA trans-10,cis-12	1,68	0,06	1.50 *	0,05	1.39 *	0,05
CLA Mix	1,68	0,10	1.65 **	0,09	1.52 **	0,06

[1]Absorbance was measured at 450 nm, and data are reported as optical density (OD_{450}).

*P < 0.05; **P < 0.01: mean values were significantly different from those of the control group.

2.5 Discussion

The present study investigated the effect of the main biologically active CLA isomers, c9- t11 and t10,c12 CLA, on the cellular antioxidant response of bovine mammary gland epithelial cells (BME-UV1) and examined whether CLA isomers could play a role in cell protection against oxidative stress.

CLA supplementation increased their concentration in the cells, and BME-UV1 cultured with CLA treatments did not produce differences in uptake rates of different CLA isomers. Therefore, the uptake rate of the tested fatty acids was not influenced by their geometric structures. Results reveal that mammary cells readily picked up the tested fatty acids. Accumulation of total CLA metabolites in CLA treated cultures were time dependent and the main metabolites were 18:3 isomers (C18:3 c6c9t11 and C18:3 c6t10c12). To date, this is the first time that metabolites of delta-6-desaturation of CLA isomers are identified in bovine mammary epithelial cells. These findings are in agreement with Meadus et al. (2014) who identified metabolites of t10,c12 and c9,t11 CLA after their addiction to 3T3-adipocyte cultures and observed a delta-6-desaturase (D6D~Fads2) activity, which added a cis-6 double bond to the CLA isomers to make 18:3 isomers. Those authors reported that the different CLA treatments formed unique metabolites and the c9,t11 CLA isomer was preferentially converted by delta-6 desaturation (FADs2) followed by elongase (Elov5) to a conjugated 20:3-c8,c11,t13. Alternatively, the t10,c12 CLA was only desaturated to conjugated 18:3-c6,t10,c12, possibly being an unsuitable substrate for further elongation (Banni et al., 2004). In rodents and in human, also metabolites originated by peroxisomial beta-oxidation of CLA

isomers have been reported (Banni et al., 2004). In the present study, conjugated 16:2 isomers were not identified, probably due to the short time of incubation. To reduce oxidative damage, the cells modulate the redox status as a cytoprotective strategy. Results of the present study clearly showed that the exposure of cells to 50 μM CLA isomers for 48 h produced a significant intracellular GSH increase, matched by high concentration of NADPH and an increase of γ-glutamylcysteine ligase activity. This was mainly observed in cells treated with t10,c12 CLA isomer. It was difficult to understand the low concentration of NADPH in CLA Mix treated cells. In this study, CLA ability to elicit cytoprotective effects (enhancement of cellular GSH and NADPH availability, and γGCL activity) was shown for the first time in bovine mammary gland, and this is in agreement with literature data showing the antioxidant potential property of CLA (Jang et al. 2004; Fagali and Catalá 2008; Chinnadurai et al., 2013). Similar results were obtained also by Arab et al. (2006) in an in vitro study using human skin fibroblasts treated with eight different fatty acids, including c9,t11 CLA. In that study, GSH synthesis was upregulated by c9,t11 CLA, mainly through an induction of γGCL. GSH plays a primary role in the maintenance of intracellular redox homeostasis by affording protection against reactive oxygen and nitrogen species as well as electrophilic xenobiotics. Under normal cellular redox conditions, the major portion of this redox regulator is in the form of GSH, whereas GSSG concentration increases during oxidative stress. Replenishment of depleted GSH levels during oxidative stress, by de novo synthesis, is of great physiological relevance due to the multiplicity of the functional roles of GSH (Weijl et al., 2004). Its homeostasis is modulated to a large extent by two enzymes: γ-glutamylcysteine cysteine ligase, which catalyzes the committed step in GSH biosynthesis, and γ-glutamyltranspeptidase, which initiates GSH reclamation. Above all an induction of γGCL is characteristic of an antioxidant response (Lee et al. 2003; Rahman, 2005). Moreover NADPH is essential in GSH recycling and related antioxidant functions (Rinaldi et al., 2004) and the ratio of the reduced to total pyridine nucleotide pool is generally accepted as an indicator of the cellular redox status (Adams et al., 2001). Toroser et al. (2006) reported that NADPH has regulatory effects on γGCL activity and increased maximal γGCL activity by up to 93% in the kidney of rats, so it may be possible to modulate de novo GSH synthesis and to control GSH homeostasis using amounts of pyridine dinucleotide phosphates.

Based on results of the present study with enhanced GSH and NADPH availability and γGCL activity, we suggest that in BME-UV1 cells the upregulated GSH synthesis by CLA

may be determined by the induction of γGCL by NADPH, mainly in cells treated with t10,c12 CLA isomer.

These findings are consistent with literature data indicating enhanced GSH content via the enhanced activation and induction of γGCL in human skin fibroblast and in MRL/MpJ-Fasl mice after CLA administration (Arab et al. 2006; Bergamo et al. 2006; Bergamo et al. 2007). In particular, Arab et al. (2006) clearly demonstrated that only CLA and no other unsaturated fatty acids were responsible in improving GSH synthesis.

In addition to non-enzymatic antioxidants as GSH the cells can attenuate the effect of oxidative stress by antioxidant enzyme system such as superoxide dismutase and various GSH-related enzymes (GPx1, GST and GR). In the present study, CLA isomers induced an increase of SOD, GPx1and GST activity and a decrease of GR activity. Hence, enhanced the activity of SOD, GPx1, GST activity the CLA treatment may offer higher protection to the cells against oxidants.

Similar results were reported by *in vivo* studies on laboratory animals fed with CLA at high levels (Ip et al., 1991; Santos Zago et al., 2007; Chinnadurai et al., 2013). Those studies showed that administration of CLA to the diet of rats increased catalase, SOD and GST enzyme activities, which finally lead to an improvement in the antioxidative process.

The decrease of GR activity induced by CLA treatment may be ascribed to the higher presence of reduced GSH in the cells possibly due to de novo GSH synthesis by GCL; so it is not necessary GSSG recycling to GSH.

The absence of gene expression modifications in antioxidant enzymes suggests that the CLA isomers improve the antioxidative enzyme systems through a post-transcriptional regulation. Sadi (2010) reported that redox sensitive proteins execute their functions via kinases, phosphatases, and transcription factors influencing the steady state levels of antioxidant enzymes. Therefore, cells may sense, transduce, and translate the oxidant signals into appropriate cellular responses depending on the cellular redox state.

Other than antioxidants, we also measured ROS and TBARS. Results indicated that the presence of CLA was associated with a low induction of ROS production. Therefore, high GSH content maintains the redox status of the cell, in this way, it is related to low intracellular ROS levels. In addition, CLA induced GSH synthesis, without any induction of ROS production. As the ability of CLA to enhance GSH content relies on γGCL induction, and the main inducers of this activity are ROS, PPAR-γ and NADPH (Wild et al., 1999; Toroser et al., 2006). These results confirm the hypothesis that the induction of γGCL by

CLA might be mediated through NADPH or, as suggested also by Arab et al. (2006), through the activation of transcription factors of PPARs.

TBARS were chosen because they have been hypothesized to represent a composite number of lipid oxidative-end products, including malondialdehyde. Therefore, the measurement of TBARS is indicative of lipid peroxidation and a biomarker of oxidative damage. In this study CLA isomers supplementation decreased cellular concentration of TBARS. Thus, the association of this biomarker with low ROS induction seems to confirm the improvement of cell oxidative status. Fagali and Català (2008) clearly indicated that CLA isomers and not linoleic acid or its methyl ester provide protection against free radicals. Finally, the measurement of cell viability in the presence of H_2O_2 was used as a further test to check cell protection. From results of the present study it is clear that CLA protected cells against oxidative damage induced by H_2O_2, and this protection seems to be mediated by the high level of GSH. In addition, Arab et al. (2006) reported that among eight fatty acids tested, CLA was the only PUFA able to have a protective action against oxidative insult in human skin fibroblasts. The protective effect of a CLA-rich diet has already been described in laboratory animals (Chinnadurai et al., 2013) and the potential improving of energy balance and metabolic status observed in bovine fed with CLA-rich diets (van Knegsel et al., 2014) could be also related to the enhancement of the thiol redox status described here.

2.6 Conclusions

Findings of the present study corroborate an antioxidant role of CLA, in particular of t10,c12 CLA isomer, by developing a significant elevated redox status in cells. In the present study, we showed that CLA isomers are potent inducers of GSH synthesis. The induction of γGCL by CLA could be mediated through NADPH and CLA has a protective action due to GSH synthesis without lipoperoxidation. Moreover, the enhancement of anti-oxidative capacity and cell protection against oxidative damages in mammary cells may be due to a direct effect of CLA on cell integrity.

The potential role of CLA in improving energy balance and metabolic status observed in dairy cow might be also related to the enhancement of the thiol redox status described here. Moving from these findings, we propose that under physiological stress situations, as the periparturient period, the improvement of the intracellular redox homeostasis by CLA treatment could be a preparative mechanism against the relative oxidative stress caused by

energy imbalance due to nutritional status, adipose mobilization, and changes in hormone profiles.

Based on the data presented here, further *in vivo* studies are necessary to fully understand the potential role of CLA on the metabolism and antioxidant capabilities of transition cows and to show how these CLA isomers may reduce incidence of oxidative stress and related diseases in early lactation.

2.7 References

Adams, J. D. Jr., L. K. Klaidman, M. L. Chang, and J. Yang. 2001. Brain oxidative stress-Analytical chemistry and thermodynamics of glutathione and NADPH. Curr. Top. Med. Chem. 1:473–482.

Andreoli, M. F., P. G. Illesca, M. A. González, and C. A. Bernal. 2010. Conjugated linoleic acid reduces hepatic steatosis and restores liver triacylglycerol secretion and the fatty acid profile during protein repletion in rats. Lipids 45:1035–1045.

Arab, K., A. Rossary, L. Soulère, and J. P. Steghens. 2006. Conjugated linoleic acid, unlike other unsaturated fatty acids, strongly induces glutathione synthesis without any lipoperoxidation. Br. J. Nutr. 96:811–819.

Banni S., A. Petroni, M. Blasevich, G. Carta, L. Cordeddu, E. Murru, M. P. Melis, A. Mahon, and M. A. Belury. 2004. Conjugated linoleic acids (CLA) as precursors of a distinct family of PUFA. Lipids. 39:1143-1146.

Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Ann. Rev. Nutr. 23:203–227.

Bauman, D. E., J. W. I. Perfield, K. J. Harvatine, and L. H. Baumgard. 2008. Regulation of fat synthesis by conjugated linoleic acid: lactation and the ruminant model. J. Nutr. 138:403–409.

Baumgard, L. H., B. A. Corl, and D. E. Bauman. 2000a. Effect of CLA isomers on fat synthesis during growth and lactation. Proc. Cornell Nutr. Conf. Feed Manuf., Ithaca, NY: Cornell Nutrition Conference (62nd). Pp. 180–190.

Baumgard, L. H., B. A. Corl, D. A. Dwyer, A. Saebø, and D. E. Bauman. 2000b. Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis. Am. J. Physiol. Regul. Integr. Comp. Physiol. 278:R179–R184.

Bergamo, P., D. Luongo, F. Maurano, G. Mazzarella, R. Stefanile, and M. Rossi. 2006. Conjugated linoleic acid enhances glutathione synthesis and attenuates pathological signs in MRL/MpJ-Fas (lpr) mice. J. Lipid Res. 47:2382–2391.

Bergamo, P., F. Maurano and M. Rossi. 2007. Phase 2 enzyme induction by conjugated linoleic acid improves lupus-associated oxidative stress. Free Radic. Biol. Med. 43:71–79.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J. Dairy Sci. 88:2017–2026.

Castañeda-Gutiérrez, E., T. R. Overton, W. R. Butler, and D. E. Bauman. 2005. Dietary supplements of two doses of calcium salts of conjugated linoleic acid during the transition period and early lactation. J. Dairy Sci. 88:1078–1089.

Chen, C.N., H.M. Brown-Borg, S. G. Rakoczy, D. A. Ferrington, and L. V. Thomposon . 2010. Aging impairs the expression of the catalytic subunit of glutamate-cysteine ligase in soleus muscle under stress. J. Gerontol. A. Biol. Sci. Med. Sci. 65:129–137.

Chinnadurai, K., H. K. Kanwal, A. K.Tyagi, C. Stanton, and P. Ross. 2013. High conjugated linoleic acid enriched ghee (clarified butter) increases the antioxidant and antiatherogenic potency in female Wistar rats. Lipids Health Dis. 12:121.

Da Silva Marineli R., A. y Castro Marques, C. P. Busch Furlan, and M. R. Maróstica Jr. 2012. Antioxidant effects of the combination of conjugated linoleic acid and phytosterol supplementation in Sprague–Dawley rats. Food Res. Int. 49:487-493.

De la Torre, A., E. Debiton, D. Durand, J. M. Chardigny, O. Berdeaux, O. Loreau, C. Barthomeuf, D. Bauchart, and D. Gruffat. 2005. Conjugated linoleic acid isomers and their conjugated derivatives inhibit growth of human cancer cell lines. Anticancer Res. 25:3943–3949.

den Hartigh L. J., C. Y. Han, S. Wang, M. Omer, A. Chait . 2013. 10E,12Z-conjugated linoleic acid impairs adipocyte triglyceride storage by enhancing fatty acid oxidation, lipolysis, and mitochondrial reactive oxygen species. J. Lipid Res. 54:2964-2978.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J. Dairy Sci. 82:2259–2273.

Fagali, N., and A. Catalá. 2008. Antioxidant activity of conjugated linoleic acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH techniques. Biophys. Chem. 137:56–62.

Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

Goff, J. P., and R. L. Horst. 1997. Physiological changes at parturition and their relationship to metabolic disorders. J. Dairy Sci. 80:1260–1268.

Grummer, R. R. 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. J. Dairy Sci. 76:3882–3896.

Hötger K, H. M. Hammon, C. Weber, S. Görs, A. Tröscher, R. M. Bruckmaier, and C. C. Metges. 2013. Supplementation of conjugated linoleic acid in dairy cows reduces endogenous glucose production during early lactation. J. Dairy Sci. 96:2258-2270

Huang, Z. Z., C. Chen, , Z. Zeng, H. Yang, J. Oh, L. Chen, and S. C. Lu. 2001. Mechanism and significance of increased glutathione level in human hepatocellular carcinoma and liver regeneration. FASEB J. 15:19–21.

Ip C., S. F. Chin, J. A. Scimeca, and M.W. Pariza. 1991. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Res. 51:6118–6124.

Jang, I. S., H. Y. Yang, and Y. H. Ko. 2004. Effect of conjugated linoleic acid on intestinal and hepatic antioxidant enzyme activity and lipid peroxidation in broiler chickens. Asian Australas. J. Anim. Sci. 17:1162–1167.

Keating A. F., F. Q. Zhao, K. A. Finucane, D. R. Glimm, and J. J. Kennelly. 2007. Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression. Domest. Anim. Endocrinol. 34:284-292.

Lee J. M., M. J. Calkins, K. Chan, Y.W. Kan, and J. A. Johnson. 2003. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. J. Biol. Chem. 278:12029–12038.

Mackle, T. R., J. K. Kay, M. J. Auldist, A. K. H. McGibbon, B. A. Philpott, L. H. Baumgard, and D. E. Bauman. 2003. Effects of abomasal infusion of conjugated linoleic acid on milk fat concentration and yield form pasture-fed dairy cows. J. Dairy Sci. 86:644–652.

Meadus, W. J., P. Vahmani, P. Duff, D. C. Rolland, T. D. Turner, and M. E. Dugan. 2014. Metabolism of CLA isomers, c9,t11 and t10,c12, in adipocyte cultures and their effect on delta-6 desaturase expression. In Proc. 10th World Congress on Genetics Applied to Livestock Production. Vancouver (BC), Canada, August 17-22, 2014 (Abs. n. 481).

Mele, M. C., G. Cannelli, G. Carta, L. Cordeddu, M. P. Melis, E. Murru, and S. Banni. 2013. Metabolism of c9,t11-conjugated linoleic acid (CLA) in humans. PLEFA. 89:115–119.

Melis M. P., E. Angioni, G. Carta, E. Murru, P. Scanu, S. Spada, and S. Banni. 2001. Characterization of conjugated linoleic acid and its metabolites by RP-HPLC with diode array detector. Eur. J. Lipid Sci. Technol. 103:617-621.

Odens, L. J., R. Burgos, M. Innocenti, M. J. VanBaale, and L. H. Baumgard. 2007. Effects of varying doses of supplemental conjugated linoleic acid on production and energetic variables during the transition period. J. Dairy Sci. 90:293–305.

Pappritz J., U. Meyer, R. Kramer, E . Weber, G. Jahreis, J. Rehage, G. Flachowsky, and S. Dänicke. 2011. Effects of long-term supplementation of dairy cow diets with rumen-protected conjugated linoleic acids (CLA) on performance, metabolic parameters and fatty acid profile in milk fat. Archi. Anim. Nutr. 65:89–107.

Rahman I. 2005. Regulation of glutathione in inflammation and chronic lung diseases. Mutat. Res. 579:58–80.

Sadi G., and Ö. Sadi. 2010. Antioxidants and regulation of antioxidant enzymes by cellular redox status. Turkish J. Scientific Rev. 3:95-107.

Santos-Zago L. F., A. P. Botelho, and A. C. de Oliveira. 2007. Supplementation with commercial mixtures of conjugated linoleic acid in association with vitamin E and the process of lipid autoxidation in rats. Lipids 42:845–854.

Selberg, K. T., A. C. Lowe, C. R. Staples, N. D. Luchini, and L. Badinga. 2004. Production and metabolic responses of periparturient Holstein cows to dietary conjugated linoleic acid and trans-octadecenoic acids. J. Dairy Sci. 87:158–168

Singh S. P., S. Häussler, J. F. L. Heinz, B. Saremi, B. Mielenz, J. Rehage, S. Dänicke, M. Mielenz, and H. Sauerwein. 2014. Supplementation with conjugated linoleic acids extends the adiponectin deficit during early lactation in dairy cows. Gen. Comp. Endocr. 198:13-21.

Sordillo, L. M., G. A. Contreras, and S. L. Aitken. 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Anim. Health Res. Rev. 10:53–63.

Toroser, D., C. S. Yarian, W. C. Orr, and R. S. Sohal. 2006. Mechanisms of gamma-glutamylcysteine ligase regulation. Biochim. Biophys. Acta. 1760:233-244

van Knegsel , A. T. M. , G. J. Remmelink , S. Jorjong, V. Fievez, and B. Kemp. 2014. Effect of dry period length and dietary energy source on energy balance, milk yield, and milk composition of dairy cows. J. Dairy Sci. 97:1499–1512.

von Soosten D., U. Meyer , M. Piechotta , G. Flachowsky , and S. Dänicke. 2012. Effect of conjugated linoleic acid supplementation on body composition, body fat mobilization, protein accretion, and energy utilization in early lactation dairy cows. J. Dairy Sci. 95:1222–1239.

Wang, H., H. Liu , J. Liu, K. Zhao, C. Wang, and W. Yang. 2014. High-level exogenous trans10, cis12 conjugated linoleic acid plays an anti-lipogenesis role in bovine mammary epithelial cells. Anim. Sci. J. 85:744-750.

Weijl N. I., T. J. Elsendoorn, E. G. Lentjes, G. D. Hopman, A. Wipkink-Bakker, A. H. Zwinderman, F. J. Cleton, and S. Osanto. 2004. Supplementation with antioxidant micronutrients and chemotherapy-induced toxicity in cancer patients treated with cisplatin-based chemotherapy: a randomised, doubleblind, placebo-controlled study. Eur. J. Cancer. 40:1713-1723.

Wild A. C., H. R. Moinova, and R. T. Mulcahy. 1999. Regulation of gammaglutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. J. Biol. Chem. 274:33627–33636.

Zavizion, B., M. van Duffelen, W. Schaeffer, and I. Politis. 1996. Establishment and characterization of a bovine mammary epithelial cell line with unique properties. In Vitro Cell. Dev. Biol. Anim. 32:138–148.

3. CHAPTER 2

Comparison between CLA and essential fatty acids in preventing oxidative stress in bovine mammary epithelial cells (BME-UV1)

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3.1 Abstract

Some *in vitro* and *in vivo* studies have demonstrated protective effects of conjugated linoleic acid (**CLA**) isomers against oxidative stress and lipid peroxidation. However, only a few and conflicting studies have been conducted showing the antioxidant potential of essential fatty acids (**EFA**s). The objectives of the study were to compare the effects of CLA to other EFAs on the thiol redox status of bovine mammary epithelia cells (**BME-UV1**) and their protective role against oxidative damage on the mammary gland by an *in vitro* study. BME-UV1 cells were treated with complete medium containing 50 µM of cis-9, trans-11 CLA (**c9, t11** CLA), trans-10, cis-12 CLA (**t10, c12** CLA), (α)-linolenic acid (**aLnA**), (γ)-linolenic acid **(gLnA**) and linoleic acid (**LA**). To assess the cellular antioxidant response, glutathione (GSH/GSSH), nicotinamide adenine dinucleotide phosphate (**NADPH**), and γ-Glutamyl-cysteine ligase (**γGCL**) activity were measured 48 hours after addition of fatty acids (**FAs**). Also intracellular Reactive Oxygen Species (**ROS**) and malondialdehyde (**MDA**) production were assessed in cells supplemented with FAs. ROS production after 3 h of H_2O_2 exposure was assessed in order to evaluate and to compare the potential protection of different FAs against H_2O_2-induced oxidative stress. All FAs treatments induced an intracellular GSH increase, matched by high concentration of NADPH and an increase of γGCL activity. Cells supplemented with FAs showed a reduction in intracellular MDA levels. Especially, CLA isomers and LA supplementation showed a better antioxidant cellular response against oxidative damage induced by H_2O_2 compared with other FAs.

Key words: conjugated linoleic acid; essential fatty acids; oxidative status; bovine mammary cells

3.2 Introduction

Lipids contained in dietary fat are known to be an excellent source of energy, and studies undertaken in the first quarter of the 20th Century demonstrated that they were necessary for growth and normal physiological function (Spector and Kim, 2014). In recent years there has been a great interest regarding the health properties of functional fatty acids, such as long-chain omega-3 and omega-6 fatty acids and conjugated linoleic acids, because of their

biological roles in cells (Lunn and Theobald, 2006, Bauman and Lock, 2006). Omega-6 and omega-3 fatty acids are regarded as essential (EFAs) because mammals cannot synthesized them and these compounds must be obtained from diet. EFAs form an important constituent of all cell membranes, and confer to membranes properties of fluidity and thus, determine and influence the behavior of membrane-bound enzymes and receptors (Das, 2006). Among omega-6 fatty acids linoleic acid (LA; 18:2n-6) is the most common n-6 PUFA; whereas alpha (α)-linoleic acid (aLnA), belonging to cis n-3 PUFA is the most prevalent omega-3 fatty acid. LA is often found in nature and is present in the seeds of most plants except for coconut, cocoa, and palm. aLnA, on the other hand, is found in the chloroplasts of green leafy vegetables, and in the seeds of flax, rape, chia, perilla and in walnuts (Lunn and Theobald, 2006; Simopoulos, 2008). It is from these two 'parent' EFAs that the n-3 and n-6 fatty acid 'families' are derived through a series of enzyme catalyzed desaturation and elongation reactions, that generally take place in the cell cytosol or in the mitochondria. aLnA is metabolized to docosahexaenoic acid (**DHA**; 22:6n-3) via eicosapentaenoic acid (**EPA**; 20:5n-3) and docosapentaenoic acid (**DPA**; 22:5n-3), whereas LA is metabolized to arachidonic acid (**AA**; 20:4n-6) via gamma (γ)-linolenic acid (**gLnA**; 18:3n-6) or eicosadienoic acid (20:2n-6) as two pathways are active (Lunn and Theobald, 2006). AA, the derivative of LA, can be converted into the 2-series of thromboxanes, and the 4-series of leukotrienes. These are very important, active and short-lived hormones termed "eicosanoids" which are involved in various patho-physiological processes concerning inflammatory conditions such as atherosclerosis, obesity, and inflammatory bowel disease. In contrast, the aLnA derivate, such as EPA, gives rise to an entirely different set of eicosanoids. These are the 3-series prostaglandins and thromboxanes and the 5-series leukotrienes, which are considered to be less inflammatory or even anti-inflammatory in comparison to the eicosanoid family derived from AA (Patterson et al., 2012).

In dairy cows, it has been observed that dietary LA and aLnA supplementation can be a nutritional strategy to improve reproductive performance and increase the percentage of pregnancies in lactating cows (Dirandehet al., 2014). This is because EFAs can stimulate the follicular growth and steroid production (Leroy et al., 2013). Furthermore, LA and aLnA are able to modulate the inflammation and the immune response in dairy cows (Caldari-Torres et al., 2011; Lessard et al., 2003).

Conjugated linoleic acids (CLA) are a group of constitution and conformation isomers of linoleic acid that are formed in the course of biohydrogenation in the rumen and later endogenous conversion from vaccenic acid (Bauman et al., 2008; Schmid et al., 2006). The isomers that contain a double bond in the trans configuration are biologically active. The most studied bioactive CLA are the cis-9, trans-11 isomer and the trans-10, cis-12 isomer. The role of CLA on health has been investigated in animal and human studies (Mele et al., 2013; Pires and Grummer 2008). It was observed that CLA might play an important protective role in cancer, cardiovascular diseases, obesity, osteoporosis and in the immune and inflammatory responses (Benjamin and Spener, 2009; Du et al., 2014; Moraes et al., 2012; Oliveira et al., 2012).

Ruminants have evolved feeding on fresh grass and leaves. According to their feed preferences, Hofmann and Stewart (1972) classified them as concentrate selectors, intermediate-mixed feeders or grass and roughage eaters. The dairy cow belongs to the group of grazer/roughage eaters such as *Bos primigenius* (aurochs), relying on grasses and roughage. Modern TMR-diets differ significantly from the natural diet of a cow e.g. in its ruminal production of CLAs. Elgersma et al (2004) showed that milk from cows feeding on fresh grass is quickly dropping in CLA when the diet was changed to the more modern silage/concentrate TMR-type. In a review, Elgersma et al. (2006) conclude in general that cows on fresh grass (e.g. pasture) produce milk with a significant higher level of CLA. This observed difference in CLA content in milk triggered extensive research into increasing CLA levels in milk from TMR fed cows with the focus being on human health.

However, these studies triggered only limited interest considering CLA as a nutrient and the wellbeing of the cow in the first place. Studies on farm animals showed that some CLA-isomers decrease milk fat synthesis, and so may improve energy balance and alleviate energy demands in lactating dairy cattle. It is routinely observed that CLA reduce milk fat percentage, increase milk yield, improve reproduction in early lactation, reduce incidence of metabolic disorders during early lactation (ketosis) and reduce negative effects of inflammatory processes during the periparturient period (Bauman et al., 2008; Galamb et al., 2016). Some studies have shown the protective effects of CLA isomers against oxidative stress and lipid peroxidation in animal models (Andreoli et al., 2010; Chinnadurai et al., 2013). Gessner et al. (2015) in cows and Zeitz et al. (2015) in dairy ewes demonstrated significant higher Vitamin E and A concentrations in milk from CLA supplemented animals. Changes in the oxidative metabolism occur in the transition period of dairy cows, and several

studies suggested that oxidative stress increases the susceptibility of dairy cattle to diseases (Bernabucci et al., 2005; Castillo et al., 2005; Sordillo and Aitken, 2009). Nevertheless, results regarding the effects of supplementing antioxidants (vitamins and trace elements) on dairy cows' health and performance have been inconsistent, because in most cases, the antioxidant potential of the animals was not assessed beforehand and the nutritional strategies were not planned accordingly (Abuelo et al., 2015). Therefore, reviewing the redox balance in dairy cattle could bring one step forward for establishing new nutritional strategies that could improve transition cow health.

Our recent study (Basiricò et al., 2015) showed that CLA isomers have an antioxidant role by developing a significantly high redox status in bovine mammary cells. Only a few and contradictory reports have been published regarding the protective potential of aLnA, LA and gLnA (Arab et al., 2006b). Fagali and Catala (2008) demonstrated in an in-vitro study that CLA exhibited greater free radical quenching activity than LA or aLnA.

Therefore, the aim of the current study was to compare the effects of CLA with other EFAs on the thiol redox status of cells and their protective activity against oxidative damage on mammary gland by an *in vitro* model based on bovine mammary epithelial cells (BME-UV1).

3.3 Materials and Methods

3.3.1 BME-UV1 Culture Conditions

The BME-UV1 cell line was created at the University of Vermont from primary bovine mammary epithelial cells in culture by stable transfection with SV-40 large T-antigen. BME-UV1 cells were kindly provided by Dr. Antonella Baldi, Department FENS, University of Milan, Italy. Cells were routinely cultivated into 75 cm^2 tissue culture flasks (Costar, Corning, NY, USA), in a mixture of 50% DMEM-F12, 30% RPMI-1640 and 20% NCTC-135 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS), 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 1 μg/ml insulin, 5 μg/ml transferrin, 1 μg/ml hydrocortisone, 0.5 μg/ml progesterone, 10 μg/ml L-ascorbic acid and antibiotics (penicillin 100 IU/ml; streptomycin 100 μg/ml). All medium supplements were from Sigma-Aldrich (Milano, Italy). The cells were maintained at 37°C in a humidified 5% CO_2 incubator. Cells used in the present work were at passage number between 39 and 41.

3.3.2 Experimental Design

In order to examine the role of fatty acids (FAs) on metabolic and oxidative status of bovine mammary gland, BME-UV1 cells were re-suspended in complete culture medium and after 24 h of incubation, medium was removed and cells were treated for 48 h with complete medium containing 50 μM of cis-9,trans-11 (c9-t11) CLA, trans-10, cis-12 (t10-c12) CLA, aLnA (18:3n-3), gLnA (18:3n-6) and LA (18:2n-6). Control cells were not treated. EFA were first dissolved in ethanol 95%, and the dilutions of the stock solution were made into aqueous buffers (culture medium) prior to perform biological experiments. To ensure that the residual amount of organic solvent was insignificant, since organic solvents may have physiological effects at low concentrations, a control test of cell viability was performed and no cytotoxic effect and biological differences were observed (data not shown). To test the potential protection of FAs against hydrogen peroxide (H_2O_2)-induced oxidative stress, cells were treated as described above followed by incubation at 37°C for 3 h with H_2O_2 (50 μM) and, the content of reactive oxygen species (ROS) and malondialdehyde (MDA) concentration were determined. Cell viability after 48 h from addition of FAs was determined. Reactive oxygen species and MDA were also determined in cells after 48 h exposure to single fatty acids. The fatty acids tested were purchased from Larodan (Malmo, Sweden). The experiments included at least 3 replicates per treatment and were repeated at least twice.

3.3.3 Cellular Antioxidant Response and Cell Protection

Cell viability assay. Cell viability after 48 h of incubation with FAs, was determined using XTT assay with Cell Proliferation kit II [XTT: sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; Roche Applied Science, Indianapolis, IL, USA] according to the manufacturer's instructions. Briefly, cells were seeded into 96-well microplates at an optimal density (5×10^5 cells/ml) and were incubated in the same conditions described above. For each treatment, after 48 h of exposure, 50 μl of XTT labeling mixture was added to each well. After 24 h incubation at 37°C, absorbance was measured at 450 nm.

Thiol Redox Status. The reduced (GSH) and the oxidized form of glutathione (GSSH) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) contents and the activity of γ-glutamate cysteine ligase (γGCL) were determined for assessing the thiol

redox status of BME-UV1 cells. For the determination of GSH, GSSG and NADPH, adherent cells were detached using trypsin/EDTA solution and centrifuged at 4,500 × g for 5 min. The pellet was re-suspended in 200 μL of PBS and lysed by two cycles of sonication lasted (100 W for 30 s), centrifuged (15,000 × g for 5 min at 4°C) and stored at -80°C until analysis. In cell extracts GSH/GSSH ratio and NADPH concentration were determined by colorimetric assays (BioAssay Systems, Hayward, CA, USA). Optical density was measured by a spectrophotometer at 405 and 540 for GSH/GSSG and NADPH, respectively.

The γGCL activity was determined by a fluorescence assay as described by Chen et al. (2010). The BME-UV1 cells were detached using trypsin/EDTA solution and centrifuged at 4,500 × g for 5 min at 4°C. The pellet was re-suspended in 100 μL of TES/SB buffer (wt/vol, 1/4) consisting of 20 mM Tris, 1 mM EDTA, 250 mM sucrose, 20 mM sodium borate, and 2 mM serine. The cells were sonicated at 100 W for 60 s and then centrifuged at 10,000 × g at 4°C for 10 min. The supernatants were collected and centrifuged again at 15,000 × g at 4°C for 20 min. The supernatants were collected and the protein concentrations were determined using a BCA Protein Assay Kit from Pierce (Rockford, IL, USA), with BSA used as the standard. For the γGCL activity assay, aliquots of 30 μL supernatant were mixed with 30 μL of γGCL reaction cocktail (400 mM Tris, 40 mM ATP, 40 mM l-glutamic acid, 2 mM EDTA, 20 mM sodium borate, 2 mM serine, and 40 mM $MgCl_2$). Following incubation at 37°C for 5 min, 30 μL of cysteine solution (30 mM; dissolved in TES/SB buffer) was added and the mixtures were incubated at 37°C for 13 min. The enzymatic reaction in the mixture was stopped by precipitating proteins with 200 mM 5-sulfosalicylic acid. After placing on ice for 20 min, the mixtures were centrifuged at 2,000 × g at 4°C for 10 min. Following centrifugation, 20 μL of each supernatant containing γ-glutamylcysteine (**γGC**) was added to a 96 well plate designed for fluorescence detection. For each assay, 20 μL of γGC standards, containing 5 μL of γGC reaction cocktail [5 μL of 5-sulfosalicylic acid (200 mM), 5 μL of H2O, and 5 μL of γGC standard solution (0, 20, 40, 60, 80, 100, 120, and 140 μM in TES/SB buffer)], was added to each well of the same 96-well plate to generate a standard curve. Next, 180 μL of 2,3-naphthalenedicarboxyaldehyde (**NDA**) was added to each well. Following incubation in the dark at room temperature for 30 min, the formation of NDA-γGC was measured (472 nm excitation/528 nm emission) using a fluorescent plate reader (Multimode Detector DTX 880, Beckman Coulter Inc., Fullerton, CA). The production of γGC in each sample was calculated using the standard curve. Values were expressed in μM/min/μg of total proteins.

Measurement of Malondialdehyde Production. For the determination of MDA, adherent cells were detached using trypsin/EDTA solution and centrifuged at 4,500 × g for 5 min at 4°C. The pellet was re-suspended in 200 μL of PBS and lysed by 2 cycles of sonication at 100 W for 30 s, centrifuged at 15,000 × g for 5 min at 4°C, and stored at -80°C until analysis. In cell extracts MDA concentrations were determined by colorimetric assays (Abcam, Cambridge, UK), CA). Optical density was measured by a spectrophotometer at 540 nm.

Measurement of Reactive Oxygen Species Production. To determine ROS concentration, cells were washed twice with PBS and incubated with 20 μM 2',7'-dichlorodihydrofluorescin diacetate probe (**DCFH-DA**) in PBS at 37°C for 40 min. Fluorescence was measured at 485 nm (excitation) and 535 nm (emission) wavelengths on a microplate reader (Multimode Detector DTX 880, Beckman Coulter Inc.).

3.3.4 Statistical analysis

All data of the experiment are presented as LSmeans and standard error of the means (**SEM**). The data were analyzed by ANOVA using Statistica-7 Software package (Stat Soft, Inc., USA). The significance of the differences was assessed by the Fisher's Least Square Difference (LSD) test. Significance was declared at $P < 0.05$.

3.4 Results

3.4.1 Effect of FAs on Cell Viability in BME-UV1

Cell viability was carried out using the XTT assay and was evaluated after 48 h of FAs exposure. As shown in Figure 1, no fatty acid treatments reduced the cell viability, and no differences were observed between treatments. This assay indicated that the exposure to different fatty acids did not show any cytotoxic effect.

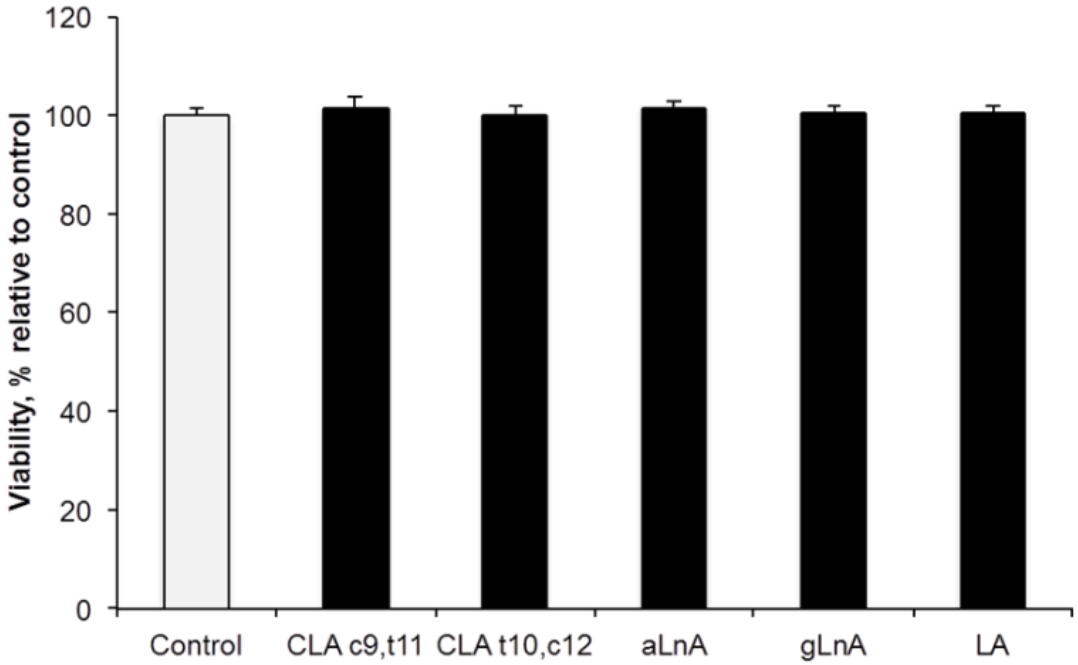

Figure 1. Cell viability of BME-UV1 cells evaluated after 48 h exposure to fatty acids. CLA = conjugated linoleic acid; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LA = linoleic acid.

3.4.2 Effect of FAs on Thiol Redox Status in BME-UV1

Oxidized and Reduced Glutathione. The levels of non-enzymatic antioxidants like GSH are depicted in Figure 2. Compared with the control, an increase ($P < 0.01$) of reduced GSH was showed in cells treated with c9,t11 CLA, aLnA, gLnA, LA, and with t10,c12 CLA ($P < 0.05$) (Figure 2A). Among treatments, values of reduced GSH were significantly higher ($P < 0.01$) in cells treated with aLnA and LA compared to c9,t11 CLA, gLnA and t10,c12 CLA. Reduced GSH level of cells treated with c9,t11 CLA and gLnA was not different between them but differed significantly ($P < 0.01$) to t10,c12 CLA. No differences in the level of oxidized GSH were noted for cells treated with FAs compared with the control culture (Figure B). The ratio GSH/GSSG showed the same trend of reduced GSH (Figure 2C).

Quantitative Determination of NADPH. Concentrations of a redox coenzyme NADPH are reported in Figure 3. Compared to the control, a greater level of NADPH was showed in cells treated with c9,t11 CLA, aLnA, gLnA, LA ($P < 0.01$) and t10,c12 CLA ($P < 0.05$). The c9,t11 CLA showed greater ($P < 0.01$) concentration of NADPH than all other FAs; the values of NADPH was not significantly different between aLnA, gLnA and LA;

whereas, NADPH in the t10,c12 CLA treated cells was significantly lower ($P < 0.05$) than cells treated with aLnA and LA.

γ-Glutamyl-Cysteine Ligase Activity. Thiol redox status was also assessed by measuring γGCL activity (Figure 4). After the 48 h, all FAs induced a strong antioxidant response by increasing ($P < 0.01$) the intracellular γGCL activity compared with the control. Among treatments, higher γGCL activity ($P < 0.01$) was observed in gLnA treatment compared with other FAs. The enzyme activity was not different between c9,t11 CLA, aLnA, and LA treatments, and was lower ($P < 0.01$) in cells treated with t10,c12 CLA compared with other FAs.

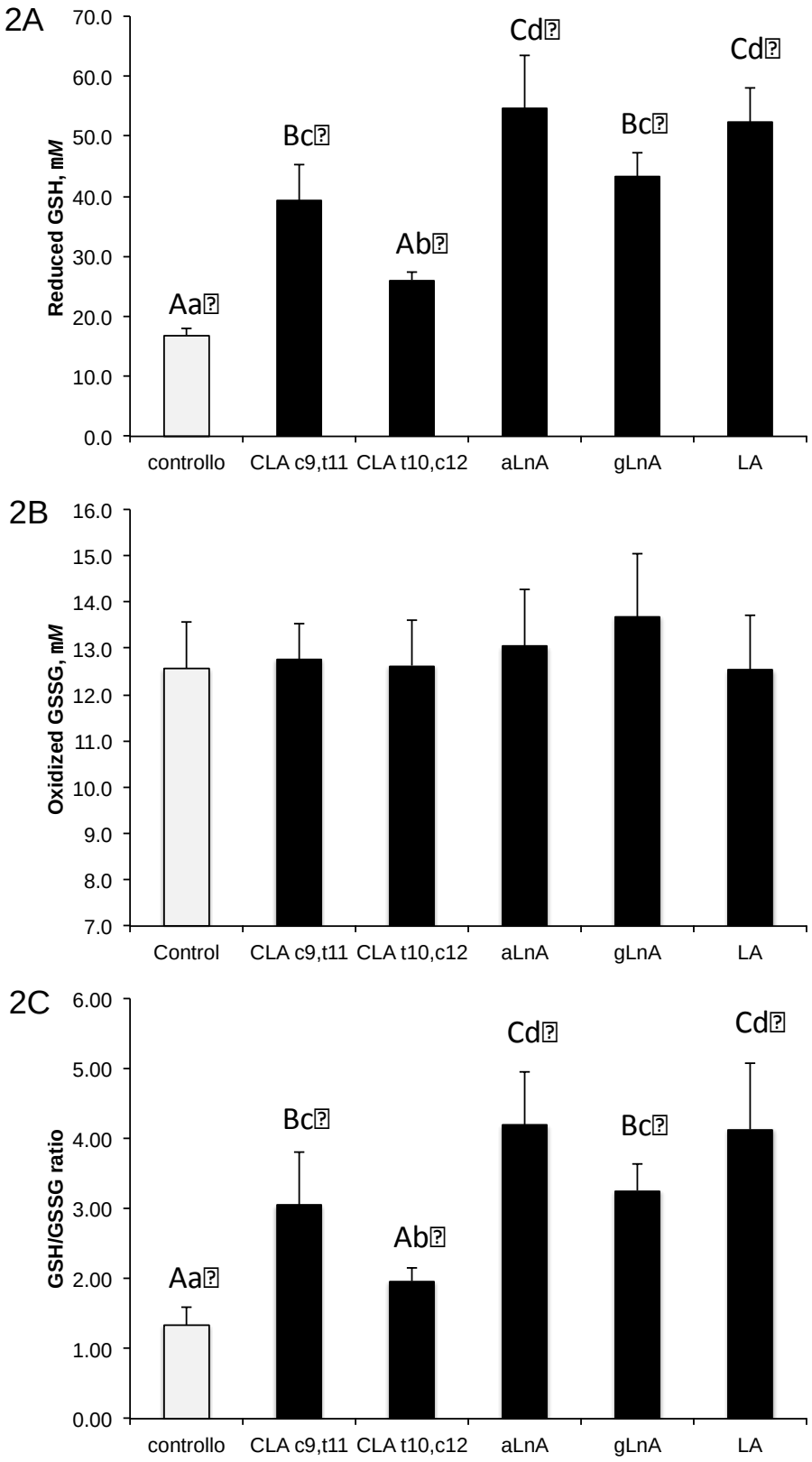

Figure 2. Intracellular concentration of reduced glutathione (GSH) (A), oxidized glutathione (GSSG) (B) and GSH/GSSG ratio (C) in BME-UV1 cells after 48 h exposure to fatty acids. Data are reported as least squares means ± SEM (n = 6). Significant differences between control and treatments and treatments between them are represented by different letters (a,b $P < 0.05$; A,B $P < 0.01$). CLA = conjugated linoleic acid; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LA = linoleic acid.

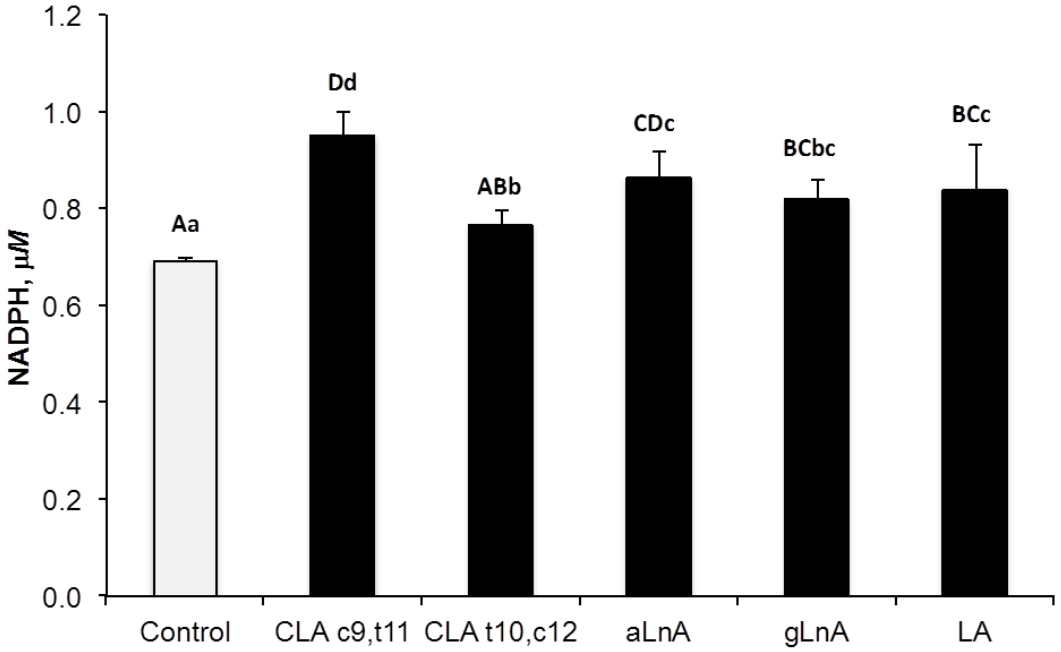

Figure 3. Intracellular concentration of nicotinamide adenine dinucleotide phosphate (NADPH) after 48 h exposure to fatty acids. Data are reported as least squares means ± SEM (n = 6). Significant differences between the control group and treatments and treatments between them are represented by different letters (a,b $P< 0.05$; A,B $P < 0.01$). CLA = conjugated linoleic acid; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LA = linoleic acid.

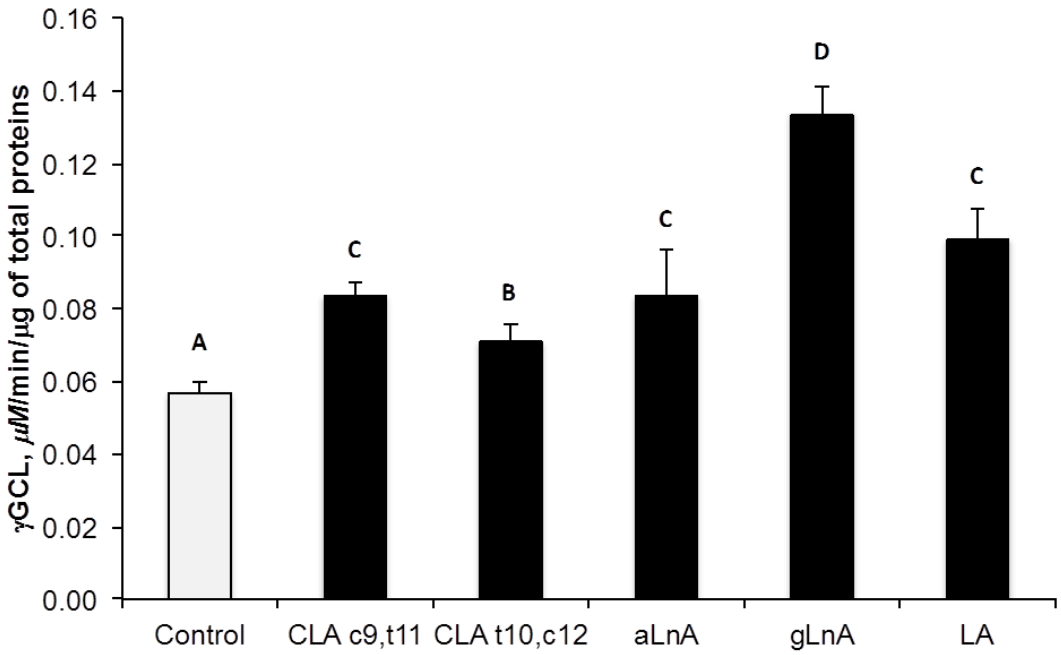

Figure 4. Intracellular activity of γ-glutamate cysteine ligase (γGCL) after 48 h exposure to fatty acids. Data are reported as least square means ± SEM (n = 6). Significant differences between control and treatments and treatments between them are represented by different letters (^{a,b} $P< 0.05$; ^{A,B} $P < 0.01$). CLA = conjugated linoleic acid; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LA = linoleic acid.

3.4.3 Intracellular ROS and MDA

Intracellular ROS production by dichlorofluorescein (DCFH-DA) fluorescence measurement was assessed in cells supplemented with FAs (Figure 5A). Cells treated with FAs showed an increase ($P < 0.01$) of ROS production compared with the control. Cells treated with gLnA showed the greatest levels ($P < 0.01$) of ROS than the other FAs. Both CLA isomers showed the same and lower levels ($P < 0.01$) of ROS production compared to other FAs treatment.

Cell concentrations of MDA are shown in Figure 5B. All FAs reduced ($P < 0.01$) the MDA level compared with untreated cells. Among treatments, only cells treated with t10,c12 CLA showed MDA levels much more higher ($P < 0.01$) than the other FAs.

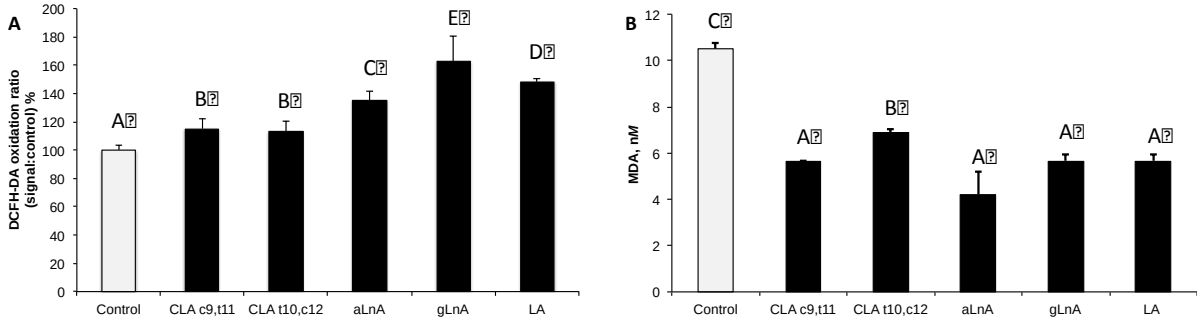

Figure 5. Intracellular production of reacting oxygen species (ROS; A) and malondialdehyde (MDA; B) in BME-UV1 cells after 48 h exposure to fatty acids. Data are reported as least square means ± SEM (n = 6). Significant differences between control and treatments and treatments between them are represented by different letters (A,B,C,D $P < 0.01$). CLA = conjugated linoleic acid; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LA = linoleic acid.

3.4.4 Effect of FAs on Protection of Cells Against Oxidative Stress

To evaluate and to compare the potential protection of different FAs against H_2O_2-induced oxidative stress, ROS test after 3 h to H_2O_2 exposure was assessed. As shown in figure 6, all FAs were not able to enhance cell resistance against oxidative stress at 48 h. The CLA treated cells showed a ROS production similar to the control and significantly lower ($P < 0.01$) than the other FAs. The ROS production was greater ($P < 0.01$) in cells treated with, aLnA, gLnA and LA compared to the control. Cells treated with gLnA showed a greater ($P < 0.01$) concentration of ROS compared to the other FAs. All FAs showed lower ($P < 0.01$)

MDA levels compared with control cells (Figure 7). Among FAs, aLnA and gLnA showed higher ($P < 0.05$) MDA levels compared with their counterparts.

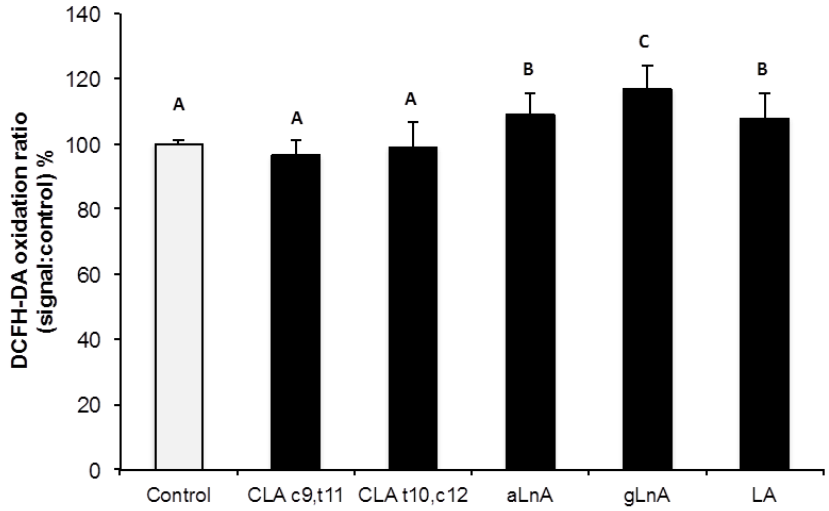

Figure 6. Intracellular production of reacting oxygen species (ROS). After 48 h exposure to fatty acids cells were treated for 3 h with H_2O_2 (50 μM). Data are reported as least squares means ± SEM (n = 6). Significant differences between the control group and treatments and treatments between them are represented by different letters (A,B $P < 0.01$). CLA = conjugated linoleic acid; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LA = linoleic acid.

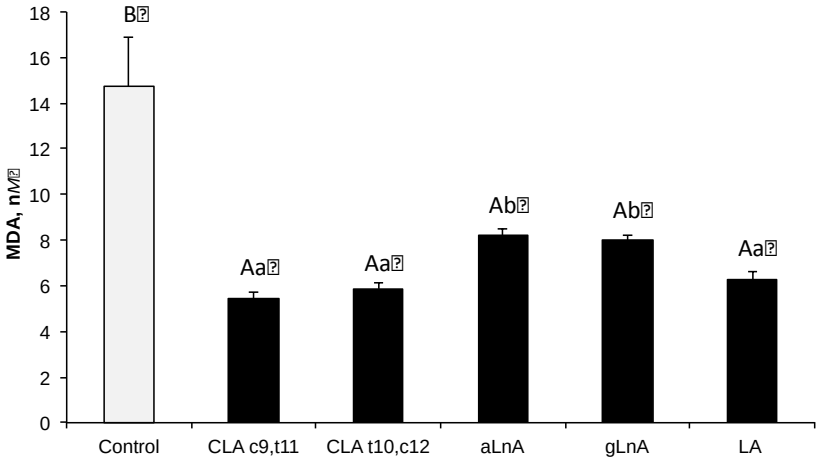

Figure 7. Intracellular production of malondialdehyde (MDA). After 48 h exposure to fatty acids cells were treated for 3 h with H_2O_2 (50 μM). Data are reported as least squares means ± SEM (n = 6). Significant differences between the control group and treatments and treatments between them are represented by different letters (a,b $P < 0.05$; A,B $P < 0.01$). CLA = conjugated linoleic acid; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LA = linoleic acid.

3.5 Discussion

In the present report we investigated the effect of several fatty acids (c9,t11 CLA, t10,c12 CLA, aLnA, gLnA, and LA) on the cellular antioxidant response and on lipoperoxidation in BME-UV1 cells. Our results have shown that the bovine cell line BME-UV1 has different susceptibilities to treatment with different EFAs and CLA.

In our experimental conditions, the exposure to 50 µM of FAs for 48 hours has strongly strengthened the cellular defenses against to oxidative damage compared with control cells. We observed that the cell supplementation of FAs induced higher levels of reduced GSH, matched by high activity of γGCL and an increase of NADPH concentration; in particular, GSH synthesis was upregulated by different PUFA, mainly through an induction of γGCL. The data presented here are consistent with our previous study (Basiricò et al., 2015) and with other findings indicating an improved antioxidant status in cells treated with CLA and EFAs (Arab et al., 2006b; Espinoza-Diez et al., 2015).

GSH plays a primary role in the maintenance of intracellular redox homeostasis by affording protection against reactive oxygen and nitrose species as well as electrophilic xenobiotics. Under normal cellular redox conditions, the major portion of this redox regulator is in the form of GSH, whereas GSSG concentration increases during oxidative stress. GSH is synthesized from its constituent aminoacids forming a tripeptide thiol and this synthesis requires two ATP-dependent steps. The first and limiting synthesis is catalyzed by γGCL and the second step is mediated by GSH synthetase. Above all an induction of γGCL is characteristic of an antioxidant response (Lee et al., 2003; Rahman, 2005). Moreover, NADPH is essential in GSH recycling and related antioxidant functions, and the ratio of the reduced to total pyridine nucleotide pool is generally accepted as an indicator of the cellular redox status (Adams et al., 2001).

In vivo study on rats fed with oils having high levels of aLnA improved the antioxidant status of hepatic tissue with the increase of GSH content and the GSH/GSSG ratio without modification of the levels of GSSG (Rincón-Cervera et al., 2016). Ramos and Colquhoun (2003), observed that gLnA and EPA in rat C6 glioma cell increased NADPH production, permitting the maintenance of adequate intracellular reduced glutathione concentrations and limiting rates of lipid peroxidation and reactive oxygen species generation.

Bergamo et al. (2011) reported that isomeric mixture of CLA (c9,t11 and t10,c12) administration in MGFD mice resulted in a significant enhancement of GSH content, up-regulated the expression of the γGCL mRNA and protein. The up-regulation of γGCL and glutathione reductase was observed in human fibroblast cells treated for 24 and 48 h with DHA (n-3 PUFA) (Arab et al., 2006a). Mollica et al. (2014) showed that c9,t11 CLA supplementation to rats improves the GSH/GSSG ratio in the liver, and nuclear factor erythroid 2 [NF-E2] - related factor 2 (**Nrf2**) activity. Cui et al., (2016) in primary mouse hepatocytes treated with different fatty acids (saturated and unsaturated, including LA, aLnA, and AA) for 24 h observed an enhancement of Nrf2 transcription factor at mRNA and protein levels. Bergamo et al. (2008) in murine dendritic cells demonstrated the c9,t11 CLA's ability to activate cytoprotective mechanism induced via Nrf2/ARE (Antioxidant Response Element).

These results provide a basis for the hypothesis that in our experiments the capacity of FAs to positively stimulate the availability of antioxidant enzyme could be related to the ability in activating the Nrf2 transcription factor. The Nrf2 pathway is the major regulator of cytoprotective responses to oxidative and electrophilic stress (Abuelo et al., 2015; Espinoza-Diez et al., 2015). Under basal conditions, Nrf2 is sequestered in the cytosol by a Keap1 homodimer, which facilitates the ubiquitination and proteasomal degradation of Nrf2. Inducers react with specific cysteine residues in Keap1, leading to the release of Nrf2 and all owing its nuclear translocation. In the nucleus, Nrf2 heterodimerizes together with small Maf proteins binds to the ARE, activating the expression of a battery of cytoprotective genes, such as NAD(P)H:quinone oxidoreductase, glutathione S-transferases, and γGCL (Lee et al., 2003).

On BME-UV1 cells, in addition to the evaluation of antioxidants, ROS amount and MDA concentration were measured. Although the amount of free radicals was higher in all cells treated with FAs compared to the control, the cells treated with c9,t11 CLA and t10,c12 CLA showed lower levels of ROS production than the other FAs. Espinoza-Diez et al. (2015) reported that low or moderate concentrations of ROS, historically considered as cellular damaging agents, are also involved in physiological responses as part of signaling processes, like activation/deactivation cycles of redox metabolism and expression of antioxidant enzymes expression that are essential to maintain oxidant/antioxidant balance in the cells. In fact, ROS protects the cell against ROS damage by inducing different antioxidant responses and re-establishing or maintaining redox homeostasis. Moreover, ROS can activate Nrf2 (Nair

et al., 2008), which in turn counteracts pro-inflammatory signaling pathways (Kim et al., 2010). These results support the hypothesis that the treatment of BME-UV1 with FAs might initiate cellular antioxidant responses through Nrf2-mediated activation of antioxidant and detoxifying enzymes.

MDA concentration has been employed for some time as a biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids (Ayala et al., 2014). Lipid peroxidation can be described generally as a process under which oxidants such as free radicals, take electrons from lipids. Lipid peroxidation is a chain reaction initiated by the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of PUFA (Repetto et al., 2012). In this study, all FAs supplementation decreased the cellular concentration of MDA compared to the control. This would demonstrate a protective effect of FAs against the lipoperoxidation. In fact, the lowest presence of end products such as MDA can be translated into a lower concentration of peroxyl radical and hydroperoxil, both triggering factors of lipid oxidation. The reduced concentration of oxidizing agents may be linked to the enhanced GSH content, relies on γGCL and NADPH induction.

Finally, the measurement of ROS and MDA production in the presence of H_2O_2 were used as further tests to check and to compare the potential protection of different FAs against H_2O_2-induced oxidative stress. The protective effects of the five fatty acids studied were not identical in mammary cells, and fatty acids induced different ROS and MDA levels in bovine mammary epithelial cells. H_2O_2 challenge of cells, which were pre-incubated with LA, or aLnA or gLnA produced oxidative damage beyond the control group while the same challenge of the cells with CLA isomers did not differ from the control cells in terms of oxidative stress. This might also be explained by extracellular formed lipid(per)oxidation products.

All FAs strongly reduced the lipoperoxidation in cells after H_2O_2 exposure. CLA and LA seems to have a better protective effect compared with aLnA and gLnA. The antioxidative effect of CLA against lipoperoxidation has been recently demonstrated in lactating dairy cows (Hanschke et al., 2016).

This is an interesting finding, considering the fact that in the course of evolution cows developed the ability to alter EFAs through biohydrogenation leading to the synthesis of CLA, which may play a more significant role than the EFAs ingested from dietary sources. CLA protection seems to be mediated not only to the levels of GSH, since reduced GSH, GSH/GSSG ratio and the activity of γGCL enzyme were lower compared to the other FAs. In

addition, Arab et al. (2006b) reported that among eight FAs tested, CLA was the only PUFA able to have a protective action against oxidative insult in human skin fibroblasts. Richard et al. (2008) reported that supplementation of human aortic endothelial cells with polyunsaturated fatty acids of the omega-3 series resulted in lower formation of ROS, as compared with cells supplemented with saturated, monounsaturated, or polyunsaturated fatty acids of the omega-6 series. Furthermore, because of varying protective mechanisms by different fatty acids on intracellular oxidative stress and cell response, the expressions of ROS production, with or without H_2O_2, did not show the same variation tendency for all fatty acids. Interestingly, the results indicated that in BME-UV1 cells gLnA was more potent in inducing ROS production and MDA than other unsaturated fatty acids. A possible explanation could be that gLnA, being a precursor of AA, can be quickly utilized as inflammatory mediator.

The capability to mitigate the negative effects of H_2O_2 by CLA isomers better compared with other FAs can be explained, in part, by the fact that lactating cows have the ability to endogenously synthesize CLA. In particular the mammary gland plays a role of primary importance in the endogenous synthesis of cis-9, trans-11 CLA. Differences in cellular uptake, incorporation and metabolization of CLA compared to EFAs probably play a significant role in the effect of these different compounds on cell response. Given the results of this and of other studies, we may conclude that CLA isomers are more effective on cellular level than EFAs because they are natural and more familiar to ruminant cells.

3.6 Conclusions

In the present report, we investigated the effect of several fatty acids on the cellular antioxidant response and on lipoperoxidation in BME-UV1 cells. Results indicate that all fatty acids were able to up-regulate the γGCL activity and enhance GSH synthesis and reduce lipoperoxidation.

It is worth noting that CLA isomers supplementation, compared with other EFAs, does not contribute to further increase of H_2O_2-induced ROS production. The increase of GSH synthesis observed in cells treated with all FAs is not sufficient to control H_2O_2 production induced by H_2O_2.

Probably in this case the increased availability of GSH was used by other pathways including reactions of transferases with xenobiotics or with lipid peroxidation products. Since ruminants

have a natural supply of CLAs, further research should consider whether this feature is an evolutionary advantage for the cow.

3.7 References

Abuelo, A., J. Hernandez, J. L. Benedito, and C. Castillo. 2015. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. J. Anim. Phisiol. Anim. Nutr. 99:1003-1016.

Adams, J. D. Jr., L. K. Klaidman, M. L. Chang, and J. Yang. 2001. Brain oxidative stress-Analytical chemistry and thermodynamics of glutathione and NADPH. Curr. Top. Med. Chem. 1:473–482.

Andreoli, M. F., P. G. Illesca, M. A. González, and C. A. Bernal. 2010. Conjugated linoleic acid reduces hepatic steatosis and restores liver triacylglycerol secretion and the fatty acid profile during protein repletion in rats. Lipids 45:1035–1045.

Arab, K., A. Rossary, F. Flourie, Y. Tourneur, and J. P. Steghens. 2006a. Docosahexaenoic acid enhances the antioxidant response of human fibroblasts by upregulating γ-glutamyl-cysteinyl ligase and glutathione reductase. British J. Nutr. 95: 18–26.

Arab, K., A. Rossary, L. Soulère, and J. P. Steghens. 2006b. Conjugated linoleic acid, unlike other unsaturated fatty acids, strongly induces glutathione synthesis without any lipoperoxidation. British J. Nutr. 96: 811–819.

Ayala, A., M. F. Muñoz, and S. Argüelles. 2014. Lipid Peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Ox. Med. Cell. Longev. 1: 1-31.

Basiricò L., P. Morera, D. Dipasquale, A. Tröscher, A. Serra, M. Mele, and U. Bernabucci. 2015. Conjugated linoleic acid isomers strongly improve the redox status of bovine mammary epithelial cells (BME-UV1). J. Dairy Sci. 98:7071–7082.

Bauman, D. E. and A. L. Lock. 2006. Conjugated linoleic acid: biosynthesis and nutritional significance. In: Advances in Conjugated Linoleic Acid Research, Vol. 2, 3rd edition. Pp 93 – 136. AOCS Press. New York. Springer.

Bauman, D. E., J. W. I. Perfield, K. J. Harvatine, and L. H. Baumgard. 2008. Regulation of fat synthesis by conjugated linoleic acid: lactation and the ruminant model. J. Nutr. 138:403–409.

Benjamin, S., and F. Spener. 2009. Conjugated linoleic acids as functional food: an insight into their health benefits. Nutr. Metab. 6:1-36.

Bergamo, P., F. Maurano, R. D'Arienzo, C. David, and M. Rossi. 2008. Association between activation of phase 2 enzymes and down-regulation of dendritic cell maturation by c9,t11-conjugated linoleic acid. Immunol. Lett. 117:181-190.

Bergamo, P., M. Gogliettino, G. Palmieri, E. Cocca, F. Maurano, R. Stefanile, M. Balestrieri, G. Mazzarella, C. David, and M. Rossi. 2011. Conjugated linoleic acid protects against gliadin-induced depletion of intestinal defenses. Mol. Nutr. Food Res. 55: 248–256

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J. Dairy Sci. 88: 2017–2026.

Caldari-Torres, C., A. L. Lock, C. R. Staples, and L. Badinga. 2011. Performance, metabolic, and endocrine responses of periparturient holstein cows fed 3 sources of fat. J. Dairy Sci. 94: 1500-1510.

Castillo, C., J. Hern_Andez, A. Bravo, M. Lopez-Alonso, V. Pereira, and J. L. Benedito. 2005. Oxidative status during late pregnancy and early lactation in dairy cows. Vet. J. 169: 286–292.

Chen, C. N., H. M. Brown-Borg, S. G. Rakoczy, D. A. Ferrington, and L. V. Thomposon. 2010. Aging impairs the expression of the catalytic subunit of glutamate-cysteine ligase in soleus muscle under stress. J. Gerontol. Series A Biol. Sci. Med. Sci. 65:129–137.

Chinnadurai, K., H. K. Kanwal, A. K. Tyagi, C. Stanton, and P. Ross. 2013. High conjugated linoleic acid enriched ghee (clarified butter) increases the antioxidant and antiatherogenic potency in female Wistar rats. Lipids Health and Dis. 12:112- 121.

Cui, Y., W. Qiuju, T. Xing, and Z. Xiuying. 2016. Effects of fatty acids on CYP2A5 and Nrf2 expression in mouse primary hepatocytes. Biochem. Genet. 54:29-40.

Das, U. 2006. Biological significance of essential fatty acids. J. Assoc. Physicians India 54: 309-319.

Dirandeh, E., A. Towhidi, S. Zeinoaldini, M. Ganjkhanlou, Z. Ansari Pirsaraei, and A. Fouladi Nashta. 2014. Effects of different polyunsaturated fatty acid supplementations during the postpartum periods of early lactating dairy cows on milk yield, metabolic responses, and reproductive performances. J. Anim. Sci. 91:713-21.

Du, R., T. Zhong, W. Q. Zhang, P. Song, W. D. Song, Y. Zhao, C. Wang, Y. Q. Tang, X. Zhang, and Q. Zhang. 2014. Antitumor effect of iRGD-modified liposomes containing conjugated linoleic acid-paclitaxel (CLA-PTX) on B16-F10 melanoma. Int. J. Nanomedicine 9:3091–3105.

Elgersma, A., G. Ellen, H. van der Horst, H. Boer, P.R. Dekker, and S. Tamminga. 2004. Quick changes in milk fat composition from cows after transition from fresh grass to a silage diet. Anim. Feed Sci. Tech. 117: 13-27

Elgersma, A., S. Tamminga, and G. Ellen. 2006. Modifying milk composition through forage. Anim. Feed Sci. and Tech. 131: 207-225

Espinosa-Diez, C., V. Miguel, D. Mennerich, T. Kietzmann, P. Sánchez-Pérez, S. Cadenas, and S. Lamas. 2015. Antioxidant responses and cellular adjustments to oxidative stress. Redox Biology 6:183-197.

Fagali, N. and A. Catalá. 2008. Antioxidant activity of conjugated linoleic acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH techniques. Biophysical Chemistry 137: 56-62

Galamb, E., V. Faigl, M. Keresztes, Z. Csillik, A. Tröscher, P. Elek, M. Kulcsár, G. Huszenicza, H. Fébel, and F. Husvéth. 2016. Effect of pre- and post-partum supplementation with lipid-encapsulated conjugated linoleic acid on milk yield and metabolic status in multiparous high-producing dairy cows. J. Anim. Physiol. Anim. Nutr. (In press, doi:10.1111/jpn.12544).

Gessner, D. K., E. Most, G. Schlegel, K. Kupczyk, F.J. Schwarz, and K. Eder. 2015. Concentrations of retinol and tocopherols in the milk of cows supplemented with conjugated linoleic acid. J. Anim. Physiol. Anim. Nutr. 99:1039-1046

Hanschke, N., M. Kankofer, L. Ruda, M. Holtershinken, U. Meyer, J. Frank, S. Danicke, and J. Rehage. 2016. The effect of conjugated linoleic acid supplements on oxidative and antioxidative status of dairy cows. J. Dairy Sci. 99:1-13.

Hofmann, R. R, and D. R. M. Stewart. 1972. Grazer or Browser: a classification based on the stomach structure and feeding habits of East African ruminants. Marnmalia, Paris 36:226-240

Kim, Y. R., J. E. Oh, M. S. Kim, M. R. Kang, S. W. Park, J. Y. Han, H. S. Eom, N. J. Yoo, and S. H. Lee. 2010. Oncogenic Nrf2 mutations in squamous cell carcinomas of oesophagus and skin. J. Phatolo. 220:446-451.

Lee J., M. J. Calkins, K. Chan, Y. W. Kan, and J. A. Johnson. 2003. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. J. Biol. Chem. 278:12029–12038.

Leroy, J. L. M. R., R.G. Sturmey, V. Van Hoeck, J. De Bie, P.J. McKeegan, and P.E.J. Bols. 2013. Dietary lipid supplementation on cow reproductive performance and oocyte and embryo viability: a real benefit? Anim. Reprod. 10:258-267.

Lessard, M., N. Gagnon, and H. V. Petit. 2003. Immune response of postpartum dairy cows fed flaxseed. J. Dairy Sci. 86:2647–2657.

Lunn, J., and. H. E. Theobald. 2006. The health effects of dietary unsaturated fatty acids. Brit. Nut. Foundation, London, UK. 31:178–224.

Mele, M. C., G. Cannelli, G. Carta, L. Cordeddu, M. P. Melis, E. Murru, C. Stanton, and S. Banni. 2013. Metabolism of c9,t11-conjugated linoleic acid (CLA) in humans. Prostaglandins Leokot. Essent. Fatty Acids 89:115–119.

Mollica, M. P., G. Trinchese, G. Cavaliere, C. De Filippo, E. Cocca, M. Gaita, A. Della-Gatta, A. Marano, G. Mazzarella, and P. Bergamo. 2014. c9,t11-conjugated linoleic

acid ameliorates steatosis by modulating mitochondrial uncoupling and Nrf2 pathway. J. Lipid Res. 55:837-49.

Moraes, M. L., A. M. L. Ribeiro, A. M. Kessler, V. S. Ledur, M. M. Fischer, L. Bockor, D. Gava. 2012. Effect of CLA on performance and immune response of weanling piglets. J. Anim. Sci. 90:2590–2598.

Nair, S., S. T. Doh, J. Y. Chan, A. N. Kong, and L. Cai. 2008. Regulatory potential for concerted modulation of Nrf2- and NF-Kβ 1-mediated gene expression in inflammation and carcinogenesis. Brit. J. Cancer 99:2070-2082.

Oliveira, D. E., M. A. S. Gama, D. Fernandes, L. O. Tedeschi, and D. E. Bauman. 2012. An unprotected conjugated linoleic acid supplement decreases milk production and secretion of milk components in grazing dairy ewes. J. Dairy Sci. 95:1437–1446.

Patterson, E., R. Wall, G. F. Fitzgerald, R. P. Ross, and C. Stanton. 2012. Health Implications of High Dietary Omega-6 Polyunsaturated Fatty Acids. J. Nutr. Metab. 2012:1-16.

Pires, J. A. A., and R. R. Grummer. 2008. Specific fatty acids as metabolic modulators in the dairy cow. Rev. Bras. Zoot. 37:287–298.

Rahman, I. 2005. Regulation of glutathione in inflammation and chronic lung diseases. Mutat. Res. 579: 58–80.

Ramos L. K., and A. Colquhoun 2003. Protective role of glucose-6-phosphate dehydrogenase activity in the metabolic response of C6 rat glioma cells to polyunsaturated fatty acid exposure. Glia 43:149-166.

Repetto, M., J. Semprine, and A. Boveris. 2012. Lipid peroxidation: chemical mechanism, biological implications and analytical determination. Lipid Peroxidation, A. Catala (Ed.), InTech, DOI: 10.5772/45943. Available from: http://www.intechopen.com/books/lipid-peroxidation/lipid-peroxidation-chemical-mechanism-biological-implications-and-analytical-determination

Richard, D., K. Kefi, U. Barbe, P. Bausero, and F. Visioli. 2008. Polyunsaturated fatty acids as antioxidanta. Pharmacol. Res. 57:451-455.

Rincón-Cervera, M. Á., R. Valenzuela, M. C. Hernandez-Rodas, C. Barrera, A. Espinosa, M. M. arambio, and A. Valenzuela. 2016. Vegetable oils rich in alpha linolenic acid increment hepatic n-3 LCPUFA, modulating the fatty acid metabolism and antioxidant response in rats. Prostagland. Leukot. Essent. Fatty Acids. (In press, doi: 10.1016/j.plefa.2016.02.002)

Schmid, A., M. Collomb, R. Sieber, and G. Bee. 2006. Conjugated linoleic acid in meat and meat products: a review. Meat Sci. 73:29–41.

Simopoulos, A. P. 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp. Biol. Med. 233:674-688.

Sordillo, L. M., and S. L. Aitken. 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet. Immunol. Immunophatol. 128:104-109.

Spector, A. A., and H. Y. Kim. 2015. Discovery of essential fatty acids. J. Lip. Res. 56:11-21.

Zeitz, J.O., E. Most, and K. Eder. 2015. Effect of conjugated linoleic acid on concentrations of fat-soluble vitamins in milk of lactating ewes. J. Dairy Sci. 98: 7328-7334

4. CHAPTER 3

Anti-inflammatory effects of conjugated linoleic acid isomers and essential fatty acids in bovine mammary epithelial cells

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4.1 Abstract

Fatty acids are important modulators of inflammatory responses, in particular, omega-3 and omega-6 essential fatty acids (**EFAs**) and conjugated linoleic acid (**CLA**) have received particular attention for their ability to modulate inflammation. The objectives of this study were to compare the effects of CLA and EFAs on the expression of pro and anti-inflammatory cytokines and their protective efficacy against inflammatory status in mammary gland by an *in vitro* model based on bovine mammary epithelial cells (**BME-UV1**). BME-UV1 cells were treated with complete medium containing 50 µM of cis-9, trans-11 CLA (**c9,t11** CLA), trans-10, cis-12 CLA (**t10,c12** CLA), (α)-linolenic acid (**aLnA**), (γ)-linolenic acid (**gLnA**) and linoleic acid **(LA**). After 48 h by fatty acids (**FAs**) administration the cells were treated for 3 h with 20 µM of lipopolysaccharide (**LPS**) to induce inflammatory stimulus. Reactive oxygen species (**ROS**) production after treatments was assessed to verify and to compare the potential protection of different FAs against LPS-induced oxidative stress. The mRNA abundance of bovine pro-inflammatory cytokines [tumor necrosis factor-alpha (**TNF-α**), interleukin-1β (**IL-1β**), interleukin-6 (**IL-6**)], anti-inflammatory [interleukin-10 (**IL-10**)] and peroxisome proliferator receptor-α/γ (**PPARγ/α**) were determined in BME-UV1 by rt-PCR. The results showed that cells treated with FAs and LPS increased ROS production compared with control cells. Among treatments, cells treated with c9,t11 CLA and t10,c12 CLA isomers revealed significant lower levels of ROS production compared with other FAs. All FAs down-regulated the gene expression of pro- and anti- inflammatory cytokines. Among FAs, t10,c12 CLA, LA and gLnA showed an homogeneous reduction of the three pro-inflammatory cytokines and this may correspond to more balanced and efficient physiological activity and may trigger a better protective effect. The PPARγ gene expression was significantly higher in cells treated with t10,c12 CLA, aLnA and LA, whereas the PPARα gene expression levels were lower in cells treated with different FAs compared to the control. These results suggest that FAs inhibited the transcription of pro-inflammatory cytokines mainly by the up-regulation of PPARγ expression.

Keywords: cis-9,trans-11 CLA, trans-10,cis-12 CLA, bovine mammary gland cells, inflammation.

4.2 Introduction

The chronic inflammatory reactions are particularly important in the pathogenesis of several cow disorder, and, in this regard, important inflammatory responses are mainly frequent during the transition period of dairy cow (Bertoni et al., 2008). Generally, in the transition period dairy cow cannot adapt to growing demand of energy, which is necessary for

the fetal growth, calving and onset of lactation (Abuelo et al., 2015). As a consequence, non-esterified fatty acids (**NEFA**) are released into the bloodstream and used as an energy source. Increased blood NEFA concentration impacts both inflammatory responses of transition cows and also the immune function (Contreras and Sordillo, 2011; Lacetera et al., 2007; Scalia et al., 2006; Sordillo and Raphael, 2013). The increased lipomobilization modifies the concentration and composition of plasma NEFA. The predominant fats found in plasma NEFA around the time of calving are saturated fatty acids (palmitate and stearate), and the monosaturated (oleic acid). In contrast, the polyunsaturated fatty acids (**PUFAs**) are reduced, especially the concentrations of omega-3 fatty acids (Sordillo and Raphael, 2013). Lipid changes can be responsible for the changes of the composition of the immune and endothelial cells, and so may affect their functions. In this regard, some studies have observed that T-cells treated with fatty acids modulated the secretion of various cytokines, such as TNF-α, IL-1β, IL-6, interleukin-8 (**IL-8**), interleukin-2 (**IL-2**), IL-10 and Interferon gamma (**IFNγ**) (de Jong et al., 2014), and induced a pro-inflammatory cascade by activating toll-like receptors and modulating the production of adipokines (de Heredia et al., 2012).

Inflammatory state of the transition cow is also induced by oxidative stress which frequently occurs in this physiological phase (Bernabucci et al., 2002; 2005) and the excessive lipolysis of the animals in a state of negative energy balance (**NEBAL**) accentuates the production of reactive oxygen species (**ROS**) through the process of β-oxidation (Bernabucci et al., 2005). The excessive amount of ROS would act by activating the redox-sensitive activate transcription factor (**NF-Kβ**) that increases the expression of pro-inflammatory mediators, including cytokines (Sordillo and Raphale, 2013).

Fatty acids are important modulators of inflammatory responses, in particular, omega-3 and omega-6 essential fatty acids (**EFAs**) and CLA have received particular attention. Important active and short-lived hormones termed “eicosanoids” are produced from EFAs. Those molecules are involved in various pathological processes concerning inflammatory conditions such as atherosclerosis, obesity, and inflammatory bowel disease (Patterson et al., 2012). The eicosanoids originated from omega-3 are known to have anti-inflammatory properties, whereas those derived from omega-6 have pro-inflammatory properties (Teng et al., 2014). Also CLA play an important protective role in the immune and inflammatory responses (Benjamin and Spener, 2009; Moraes et al., 2012; Oliveira et al., 2012; Zhang et al., 2014). Hontecillas et al. (2002) observed that dietary CLA-supplementation suppressed colon inflammation in pigs with bacterial-induced colitis.

Cytokines are produced by many cell populations, the predominant specialized immune cells like T-helper cells, mast cells, macrophages, endothelial cells and also in epithelial cell (Jun-Ming Zhang, and Jianxiong, 2007). Some authors (Boudjellab et al., 2000; McClenahan et al., 2005; Medina-Estrada et al., 2016) observed that cultured bovine mammary gland epithelial cells could secrete IL-1α, IL-6, and TNF-α in response to stimulation with lipopolysaccharide (**LPS**). Therefore, the aim of the current study was to compare the effects of CLA with other EFAs on the expression of pro-inflammatory cytokines and their protective efficacy against inflammatory status in mammary gland by an *in vitro* model based on bovine mammary epithelial cells (**BME-UV1**).

4.3 Materials and Methods

4.3.1 Cell Culture Conditions

The BME-UV1 cell line was created at the University of Vermont from primary bovine mammary epithelial cells in culture by stable transfection with SV-40 large T-antigen. BME-UV1 cells were kindly provided by Dr. Antonella Baldi, Department FENS, University of Milan, Italy. Cells were routinely cultivated into 75 cm^2 tissue culture flasks (Costar, Corning, NY, USA), in a mixture of 50% DMEM-F12, 30% RPMI- 1640 and 20% NCTC-135 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS), 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 Mm glutathione, 1 μg/ml insulin, 5 μg/ml transferrin, 1 μg/ml hydrocortisone, 0.5 μg/ml progesterone, 10 μg/ml L-ascorbic acid and antibiotics (penicillin 100 IU/ml; streptomycin 100 μg/ml). All medium supplements were from Sigma-Aldrich. The cells were maintained at 37°C in a humidified 5% CO2 incubator until confluence. Cells used in the present work were at passage number between 39 and 41.

4.3.2 Experimental Design

In order to test the effects of fatty acids (FAs) on inflammatory status of bovine mammary gland, BME-UV1 cells were re-suspended in complete culture medium to a concentration of 5×10^5 cells/ml and dispensed into cells seeded in culture flasks and 96-wells tissue culture plates. After 24 h of incubation at 37°C in an atmosphere of 5% CO_2, medium

was removed and cells were treated for 48 h with complete medium containing 50 μM of cis-9,trans-11 (c9-t11) CLA, trans-10, cis-12 (t10-c12) CLA, α-linolenic acid, (aLnA; 18:3*n*-3), γ-linolenic acid (gLnA; 18:3*n*-6) and linoleic acid (LA; 18:2n-6). To test the preventive efficacy of FAs against induced inflammation by LPS (from Escherichia coli 055:B5, Sigma Aldrich), cells were treated with FAs as described above followed by incubation at 37°C for 3 h with LPS. Gene expression of pro-inflammatory cytokines, peroxisome proliferator-activated receptor-γ (PPARγ) was determined.

Also cell viability after LPS addition was measured. The fatty acids tested were purchased from Larodan (Malmo, Sweden). The experiments included at least 3 replicates per treatment and were repeated at least twice.

4.3.3 Cell viability

BME-UV1 cells were seeded into 96-wells microplates at an optimal density (5×10^5 cells/ml) and were grown with different FAs for 48 h. Then cells were incubated at 37°C for 3 h with LPS (20 μ*M*). Cell viability after incubation with LPS assay was determined using Cell Proliferation kit II [XTT: sodium 30-[1-(phenylaminocarbonyl) 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; Roche Applied Science, Indianapolis, IL, USA] according to the manufacturer's instructions. For each treatment, after 3 h of exposure to LPS, 50 μl of XTT labelling mixture was added to each well. After 24 h incubation at 37°C, absorbance was measured at 450 nm. Background absorbance was subtracted from each value. Results were expressed as optical density (O.D.).

4.3.4 Measurement of Reactive Oxygen Species Production

To determine ROS concentration, cells were washed twice with PBS and incubated with 20 μM 2',7'-dichlorodihydrofluorescin diacetate probe in PBS at 37°C for 40 min. Fluorescence was measured at 485 nm (excitation) and 535 nm (emission) wavelengths on a microplate reader (Multimode Detector DTX 880, Beckman Coulter Inc.).

4.3.5 RNA isolation and real-time PCR

The mRNA abundance of bovine pro and anti- inflammatory interleukins (TNF-α, IL-1β, IL-6 and IL-10) and PPARγ/α were assayed in BME-UV1 cultured under the conditions

described above and were carried out by rt-PCR. All primers and probes used were previously published by Bionaz et al.(2008), Gonzalez et al. (2013) and Liu et al. (2012).

In order to isolate total RNA, BME-UV1 cells were seeded in cell culture flasks at the concentration of 5×10^5 cells/ml in complete medium containing 10% FBS and treated as described above. Total RNA was extracted by QIAzol Lysis Reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions and stored at -80°C. RNA was quantified using Quant-iT RNA assay Kit (Invitrogen, Carlsbad, CA, USA) and fluorescence was measured at excitation/emission of 644/673 nm. One microgram of total RNA was reverse transcribed using Quantitect reverse transcription kit (Qiagen) in a total volume of 20 µl on a PCR Express thermal cycler(Hybaid, Ashford, UK). Quantitative SYBR Green and probes real-time PCR were performed following the manufacturer's recommendations using LightCycler (Roche). To account for possible variation related to cDNA input or the presence of PCR inhibitors, the endogenous reference gene GAPDH was simultaneously quantified for each sample, and data were normalized accordingly. In Table 1 are shown the specific characteristics of primers used for the real-time PCR.

PCR products were subjected to a melting curve analysis on the LightCycler. To allow relative quantification after PCR, standard curves were constructed from the standard reactions for each target and housekeeping genes by plotting crossing point (**Cp**) values, i.e., the cycle number at which the fluorescence signal exceeds background versus log cDNA dilution. The Cp readings for each of the unknown samples where then used to calculate the amount of either the target or housekeeping relative to the standard, using the second derivative maximum method with the LyghtCycler analysis software 3.5 (Roche Applied Science).

Table 1. DNA sequences of bovine sense and antisense primers and probe used for real-time PCR analysis

Gene[1]	Primer	Temperature of annealing, °C
PPARγ Forward	AATAACGCGATTCGTTTTGG	60
PPARγ Reverse	TCCATGTCGTGGATGAGAAA	
PPARα Forward	CGGTGTCCACGCATGTGA	60
PPARα Reverse	TCAGCCGAATCGTTCTCCTAAA	
TNF-α Forward	TCTTCTCAAGCCTCAAGTAACAAGT	60
TNF-α Reverse	CCATGAGGGCATTGGCATAC	
TNF-α Probe	FAM-AGCCCACGTTGTAGCCGACATCAACTCC-TAMRA	
IL-1β Forward	TCCACCTCCTCTCACAGGAAA	58
IL-1β Reverse	CTCTCCTTGCACAAAGCTCATG	
IL-1β Probe	FAM-CACCACTTCTCGGTTCA-MGB	
IL-6 Forward	GGGCTCCCATGATTGTGGTA	60
IL-6 Reverse	GTGTGCCCAGTGGACAGGTT	
IL-6 Probe	FAM-TTCCTGGGCATTCCCTCCTCTGGT-TAMRA	
IL-10 Forward	CTTGTCGGAAATGATCCAGTTTT	60
IL-10 Reverse	TTCACGTGCTCCTTGATGTCA	
IL-10 Probe	FAM-CCACAGGCTGAGAACCACGGGC-TAMRA	
GAPDH Forward	GCATCGTGGAGGAGGGACTTATGA	60
GAPDH Reverse	GGGCCATCCACAGTCTTCTG	
GAPDH Probe	FAM-CACTGTCCACGCCATCACTGCCA-TAMRA	

[1]PPARγ= peroxisome proliferator-activated receptorα- γ, TNF-α= Tumor necrosis factor-α, IL-1β= Interleukin-1β, IL-6= Interleukin-6, IL-10= Interleukin-10, GAPDH= Glyceraldehyde 3-phosphate dehydrogenase

4.3.6 Statistical Analysis

All data of the experiment are presented as LSmeans and standard error of the means (**SEM**). The data were analyzed by ANOVA using Statistica-7 Software package (Stat Soft, Inc., USA). The significance of the differences was assessed by the Fisher's Least Square Difference (**LSD**) test. Significance was declared at $P < 0.05$.

4.4 Results

4.4.1 BME-UV1 Cell Viability

Cell viability (Figure 1) was carried out using the XTT assay and was evaluated after 3 h of LPS exposure. As showed in figure 1 LPS treatments did not modify cell viability. These results indicate that exposure to LPS, does not affect the metabolic activity of BME-UV1 through a decrease or an increase in cells viability and that the treatment does not have a cytotoxic effect.

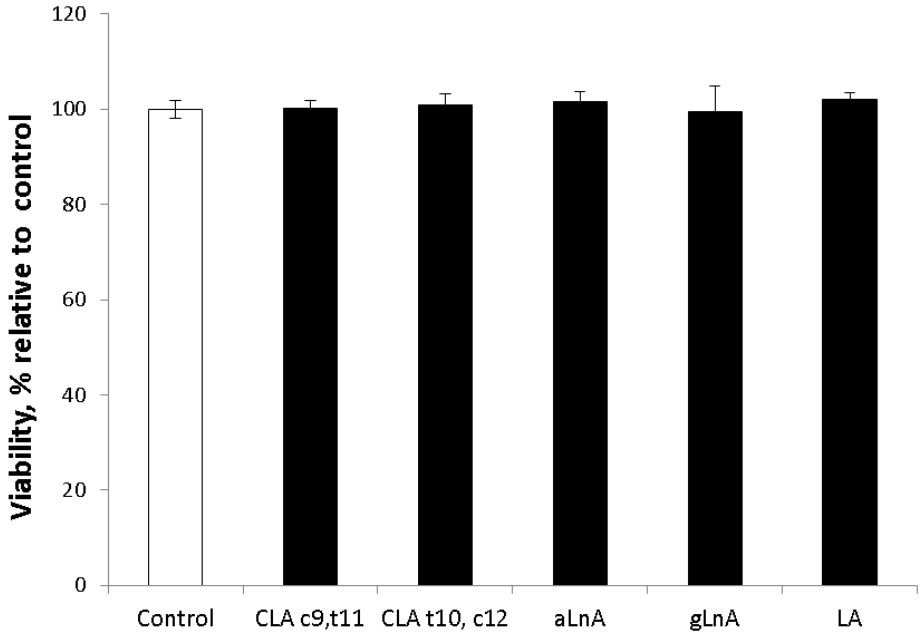

Figure 1. Cell viability of BME-UV1 cells observed, respectively, after 48 h of FAs supplementation, and after 3 h of LPS treatment (20 μM). Absorbance was measured at 450 nm, and data are reported as least squares means ± SEM (n = 6). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

4.4.2 Quantification of ROS production

Intracellular ROS production by dichlorofluorescein fluorescence measurement was assessed in cells supplemented with FAs and stimulated with LPS (Figure 2). Cells treated with FAs showed an increased ROS production ($P < 0.01$) compared to the control. Among treatments, cells treated with c9,t11 CLA and t10,c12 CLA isomers revealed significant lower levels of ROS production compared to other FAs.

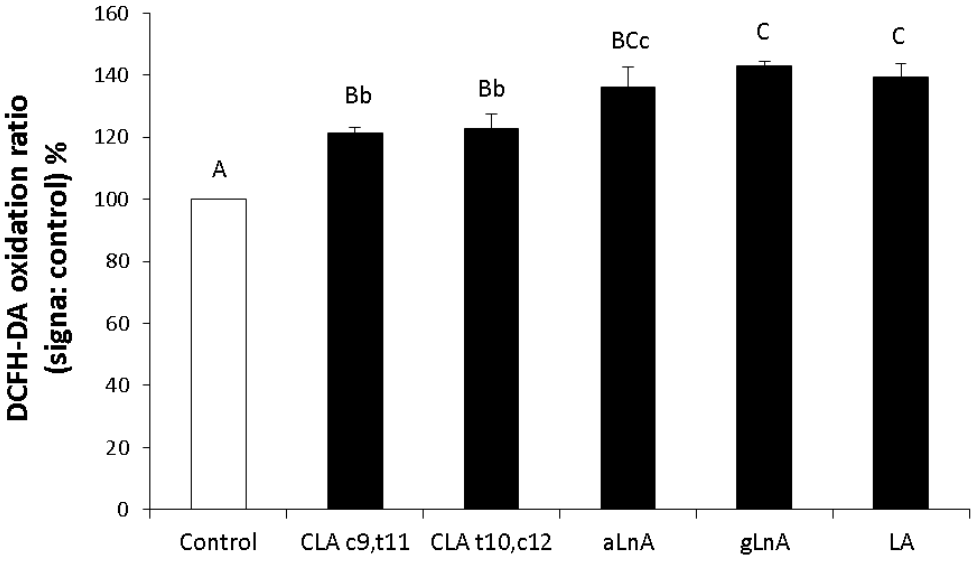

Figure 2. Intracellular production of reacting oxygen species by 2',7'-dichlorodihydrofluorescin diacetate probe in bovine mammary epithelia cells (BME-UV1) cells after 48 h of FAs supplementation, and after 3 h of LPS treatment (20 μM). Data are reported as least squares means ± SEM (n = 6). Significant differences among control and treatments are represented by different letters letters (^{a,c} $P < 0.05$; ^{A,C} $P < 0.01$). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

4.4.3 Quantification of mRNA expression of pro and anti-inflammatory cytokines

The mRNA expression of TNF-α, IL-1β, IL-6 and IL-10 are shown in Figure 3 (A, B, C and D respectively). The gene expression of pro-inflammatory cytokines was lower ($P < 0.01$) in cells treated with different fatty acids compared to the control. However, some differences were observed between the different fatty acids. In particular, the TNF-α gene expression (Figure 3.A) was lower in cell treated with t10,c12 CLA, gLnA and LA, compared with the other fatty acids and control ($P < 0.01$). Instead, c9,t11 CLA and aLnA, showed a greater TNF-α gene expression compared to the other three fatty acids but significantly lower ($P < 0.01$) than the control. The IL-1β gene expression (Figure 3.B) was lower in cells treated with c9,t11 CLA, which significantly differed to the t10,c12 CLA isomer ($P < 0.05$) and to aLnA ($P < 0.01$). IL-1β did not change between other fatty acids. The gene expression of IL-6 (Figure 2.C) was again markedly lower ($P < 0.01$) in cells treated with t10,c12 CLA, gLnA and LA, compared to c9,t11 CLA, aLnA and the control ($P < 0.01$). The gene expression of

the anti-inflammatory cytokine (Figure 3.D) was lower in cells treated with different fatty acids compared to the control. Among fatty acids, IL-10 gene expression was lower ($P < 0.01$) in cells treated with gLnA, whereas, greater level was observed in cells treated with t10,c12 CLA isomer and aLnA.

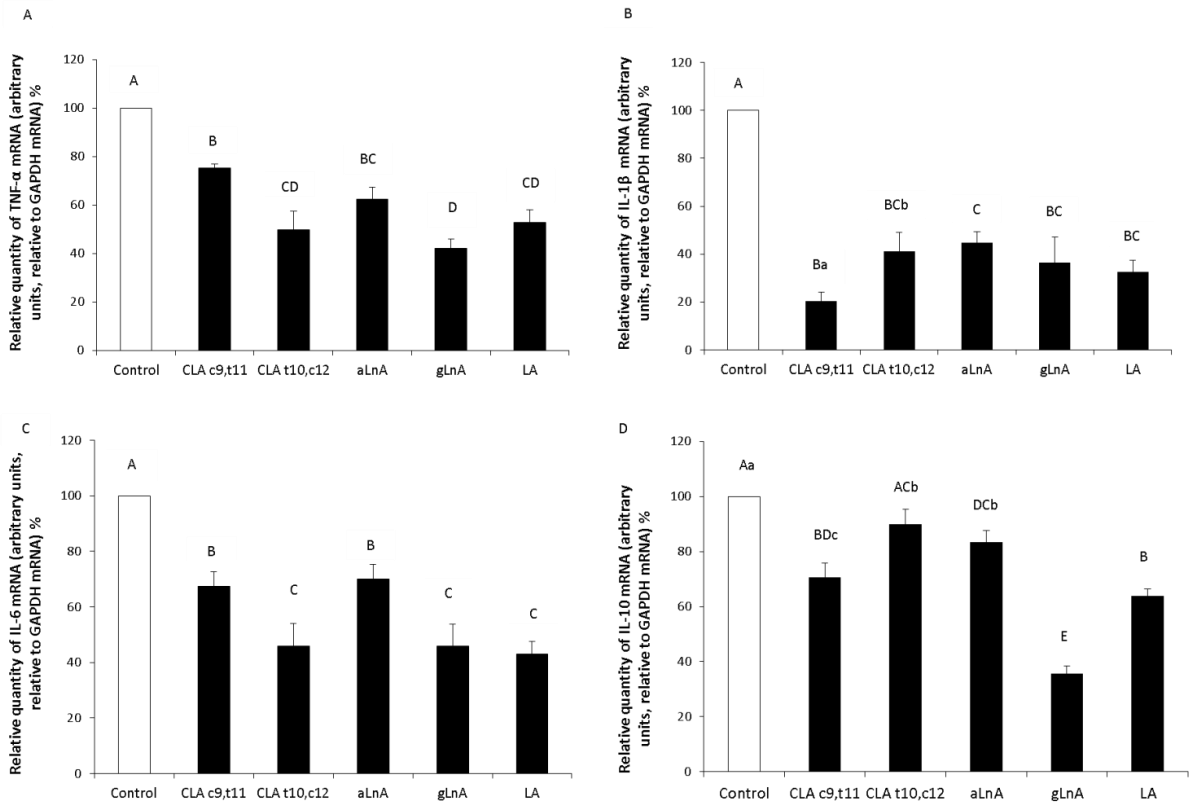

Figure 3. Messenger RNA abundance of Tumor necrosis factor-α (TNF-α; A), Interleukin-1 (IL-1β; B), Interleukin-6 (IL-6; C) and Interleukin-10 (IL-10; D) in BME-UV1 cells after 48 h of FAs supplementation, and after 3 h of LPS treatment (20 μM). Data are reported as least squares means ± SEM (n = 6). Significant differences between control and treatments and treatments between them are represented by different letters (^{a,c} $P < 0.05$; ^{A,E} $P < 0.01$). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

4.4.4 Quantification of mRNA Expression of PPARγ and PPARα

The quantification of mRNA expression of PPARγ and PPARα is showed in Figure 4 (A and B, respectively). PPARγ gene expression was significantly greater in cells treated with t10,c12 CLA, aLnA and LA compared with control, c9,t11 CLA ($P < 0.05$) and gLnA ($P < 0.01$ and $P < 0.05$). Conversely, the gene expression levels of PPARα were all lower (P-value

ranged from < 0.05 to < 0.01) in cells treated with different FAs compared to the control. Between treatments, PPARα gene expression was only lower (P < 0.05) in cells treated with c9,t11 CLA compared with LA.

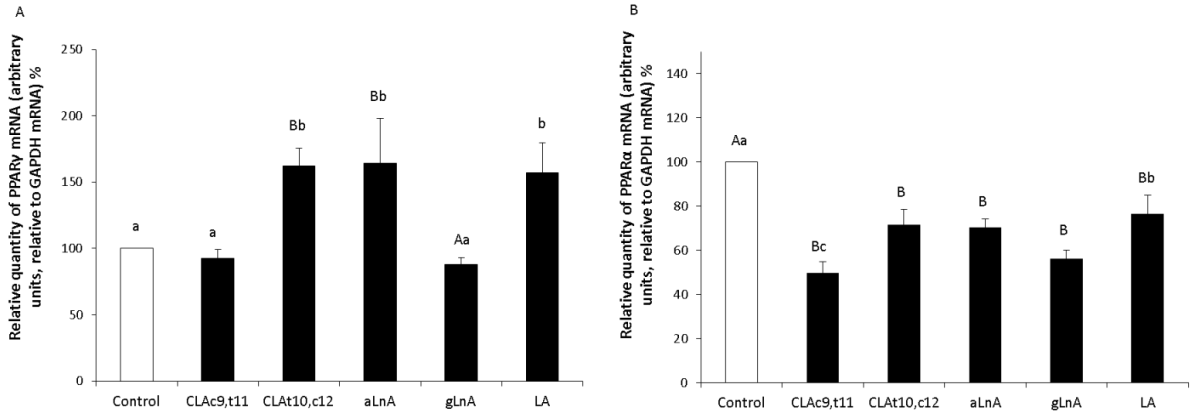

Figure 4. Messenger RNA abundance of PPARγ and PPARα in BME-UV1 cells after 48 h of FAs supplementation, and after 3 h of LPS treatment (20 μM). Data are reported as least squares means ± SEM (n = 6). Significant differences between control and treatments and treatments between them are represented by different letters $^{(a,c}$ P < 0.05; ^{A,C} P < 0.01). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

4.5 Discussion

In the present study, for the first time, the effect of several FAs (c9,t11 CLA, t10,c12 CLA, aLnA, gLnA, and LA) on the inflammatory response of BME-UV1 cells was investigated. The results showed that bovine cell line had different susceptibility to the different EFAs and CLA.

Under the experimental conditions, the exposure to 50 μM of FAs for 48 hours has protected the cells limiting the triggering of the inflammatory process induced by LPS, known as a potent endotoxin responsible to stimulate the inflammation response and inflammatory cytokine synthesis. The results of the present study showed that cell supplemented with FAs has reduced the gene expression of the main pro-inflammatory cytokines and of the anti-inflammatory cytokine IL-10, matched by increased gene expression of PPARγ. The data presented here are consistent with other findings indicating an improvement of the inflammatory status in cells treated with CLA and EFAs (Changhua et al., 2005; Erdinest et al., 2012; Lehnen et al., 2015; Zhao et al., 2007). Erdinest et al. (2012), on human corneal

epithelial cells culture incubated for 2 hours with different concentrations of aLnA, gLna and LA, showed a reduced gene expression of TNF-α, IL-1β and IL-6 in cells treated with aLnA. Those authors stated that inhibitory effect of aLnA was mediated through NF-Kβ signal transduction. Also Zhao et al. (2005) in a study on monocytic THP-1 cells pre-incubated with aLnA, LA and docosahexaenoic acid observed that FAs decreased TNF-α, IL-1β and IL-6 gene expression and the NF-Kβ DNA-binding activity, whereas PPARγ activity was increased and the anti-inflammatory properties of FAs were attributed, at least in part, to PPARγ-dependent mechanism. Changhua et al. (2005) in an *in vitro* study on the anti-inflammatory effect of c9,t11 CLA and t10,c12 CLA on cultured peripheral blood mononuclear cells, noted that CLA isomers, in particular t10,c12 CLA, suppressed the production and expression of TNF-α, IL-1 and IL-6. A recent study on primary goat mammary gland epithelial cells treated with gLnA and LA showed that gLnA but not LA reduced inflammation induced by LPS, through the inhibition of the activity of NF-Kβ transcription factor and therefore the transcription of IL-1, IL-6 and TNF-α (Cao et al., 2016). Also *in vivo* studies revealed similar results (Lee et al., 2005; Lin et al., 2013). Song et al. (2005), in humans, observed that dietary CLA supplementation reduced levels of TNF-α and IL-1β while increased the anti-inflammatory cytokine IL-10. Lin et al. (2013) showed that rats, supplemented with diet rich in omega-3 (fish oil to 5%), had lower mammary mRNA abundance of xanthine oxidoreductase, protein level of TNF-α and greater levels of IL-10 and PPAR-γ. Renner et al. (2013) showed that there were no differences in IL-10 expression in bovine peripheral blood mononuclear cells treated with c9,t11, t10,c12 CLA isomers and LA. In contrast, Verlengia et al. (2004) reported that the B-lymphocyte cell line treated with EPA and DHA reduced the production of IL-10. This suppression of cytokine production was also observed in spleen lymphocytes from mice fed fish oil-rich diets (Wallace et al., 2001). The effects of FAs on the gene expression of the anti-inflammatory IL-10 are currently ambiguous and contradictory. On the other hand, Tamayo et al. (2011) in a human study on the relationship between the responses of cytokines in septic shock, observed that the secretion of pro- and anti-inflammatory cytokines occurs in a simultaneous manner by the moment which begins the immuno-inflammatory response. These results suggest the hypothesis that the reduced anti-inflammatory cytokines gene transcription could be positively correlated with reduced transcription levels of pro-inflammatory cytokines in view of a harmonized inflammatory response.

The PPARγ transcription factor, through its activation or gene up-regulation, plays an important role in the control of inflammation (Delerive et al., 2001; Lee et al., 2005; Mandard and Patsouris, 2013). PPARγ inhibits the production of inflammatory mediators such as TNF-α, IL-1 and IL-6 interacting physically with NF-Kβ and preventing NF-Kβ translocation into the nucleus (Tak and Firestein, 2001). NF-Kβ is the main transcription factor that activate the expression of pro-inflammatory cytokines (Lawrence, 2009).

In this study it was observed an up-regulation of PPARγ in cells treated with t10,c12 CLA, aLnA and LA, but no significant differences on the expression of PPARγ were observed in cells treated with c9,t11 CLA and gLnA. Our results suggest that t10,c12 CLA, aLna and LA inhibited the transcription of pro-inflammatory cytokines through the up-regulation of PPARγ expression, whereas c9,t11 CLA and gLnA probably acted through activation of the PPARγ nuclear receptor, as observed and reported by Cao et al. (2016) and Jaudszus et al. (2005).

The levels of expression of the PPARα gene, contrarily to PPARγ, were lowest in all cells treated with the different fatty acids and stimulated with LPS compared with control. Probably this phenomenon is linked to greater production of ROS observed in FAs-treated BME-UV1 cells. Cabrero et al. (2002) observed that skeletal muscle cells treated with Etomoxir, a substance which contains a fatty acid-derived structure, showed a down-regulation of PPARα mRNA expression, through increased production of ROS and NF-Kβ activation. In addition, Cabrero et al. (2002) reported that low concentration of fatty acid-derived substance tested (50 μM) would not affect the gene expression of the PPARα target genes. It is important to remark that generation of ROS have not solely a negative effect but within certain concentrations ROS are useful signaling molecules regulating physiological processes. The beneficial or detrimental effects of ROS depends on many variables such as the site of ROS production, the persistence of ROS flow or the antioxidant status of target cells (Barbieri and Sestili, 2012).

4.6 Conclusions

All FAs reduced the gene expression of pro-inflammatory cytokines, among FAs, t10,c12 CLA, LA and gLna showed an homogeneous reduction of the three cytokines and this may correspond to more balanced and efficient physiological activity and may trigger a better

protective effect. Our results suggest that FAs inhibited the transcription of pro-inflammatory cytokines mainly by the up-regulation of PPARγ expression.

4.7 References

Abuelo, A., J. Hernández, J. L. Benedito, and C. Castillo. 2015. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. J. Anim. Physiol. Anim. Nutr. (Berl). 99:1003–1016. doi:10.1111/jpn.12273.

Barbieri, E., and P. Sestili. 2012. Reactive oxygen species in skeletal muscle signaling. J. Signal Transduct. 2012:1-17. doi:10.1155/2012/982794.

Benjamin, S., and F. Spener. 2009. Conjugated linoleic acids as functional food: an insight into their health benefits. Nutr. Metab. (Lond). 6 (36):1-13. doi:10.1186/1743-7075-6-36.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2002. Markers of Oxidative Status in Plasma and Erythrocytes of Transition Dairy Cows During Hot Season. J. Dairy Sci. 85:2173–2179. doi:10.3168/jds.S0022-0302(02)74296-3.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of Body Condition Score on Relationships Between Metabolic Status and Oxidative Stress in Periparturient Dairy Cows. J. Dairy Sci. 88:2017–2026. doi:10.3168/jds.S0022-0302(05)72878-2.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008. Effects of Inflammatory Conditions on Liver Activity in Puerperium Period and Consequences for Performance in Dairy Cows. J. Dairy Sci. 91:3300–3310. doi:10.3168/jds.2008-0995.

Bionaz, M., C. R. Baumrucker, E. Shirk, J. P. Vanden Heuvel, E. Block, and G. A. Varga. 2008. Characterization of Madin-Darby bovine kidney cell line for peroxisome proliferator-activated receptors: temporal response and sensitivity to fatty acids. J. Dairy Sci. 91:2808–13. doi:10.3168/jds.2007-0789.

Boudjellab, N., H. S. Chan-Tang, and X. Zhao. 2000. Bovine interleukin-1 expression by cultured mammary epithelial cells (MAC-T) and its involvement in the release of MAC-T derived interleukin-8. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 127:191–199.

Cao, D., J. Luo, W. Zang, D. Chen, H. Xu, H. Shi, and X. Jing. 2016. Gamma-Linolenic Acid Suppresses NF-κB Signaling via CD36 in the Lipopolysaccharide-Induced Inflammatory Response in Primary Goat Mammary Gland Epithelial Cells. Inflammation 39:1225–37.

Changhua, L., Y. Jindong, L. Defa, Z. Lidan, Q. Shiyan, and X. Jianjun. 2005. Conjugated linoleic acid attenuates the production and gene expression of proinflammatory cytokines in weaned pigs challenged with lipopolysaccharide. J. Nutr. 135:239–244.

Contreras, G. A., and L. M. Sordillo. 2011. Lipid mobilization and inflammatory responses during the transition period of dairy cows. Comp. Immunol. Microbiol. Infect. Dis. 34:281–289. doi:10.1016/j.cimid.2011.01.004.

Delerive, P., J.C. Fruchart, and B. Staels. 2001. Peroxisome proliferator-activated receptors in inflammation control. J. Endocrinol. 169:453–459.

Erdinest, N., O. Shmueli, Y. Grossman, H. Ovadia, and A. Solomon. 2012. 53(8):4396-4406 Investig. Opthalmology Vis. Sci. 53:4396. doi:10.1167/iovs.12-9724.

Gonzalez, D. D., A. Rimondi, M. S. Perez Aguirreburualde, M. Mozgovoj, D. Bellido, A. Wigdorovitz, and M. J. Dus Santos. 2013. Quantitation of cytokine gene expression by real time PCR in bovine milk and colostrum cells from cows immunized with a bovine rotavirus VP6 experimental vaccine. Res. Vet. Sci. 95:703–708. doi:10.1016/j.rvsc.2013.03.016.

Jaudszus, A., M. Foerster, C. Kroegel, I. Wolf, and G. Jahreis. 2005. Cis-9,Trans-11-CLA exerts anti-inflammatory effects in human bronchial epithelial cells and eosinophils: Comparison to Trans-10,Cis-12-CLA and to linoleic acid. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1737:111–118.

de Heredia, F. P., S. Gómez-Martínez, and A. Marcos. 2012. Obesity, inflammation and the immune system. Proc. Nutr. Soc. 71:332–338. doi:10.1017/S0029665112000092.

de Jong, A. J., M. Kloppenburg, R.E.M. Toes, and A. Ioan-Facsinay. 2014. Fatty acids, lipid mediators, and T-cell function. Front. Immunol. 5(483):1-7. doi:10.3389/fimmu.2014.00483.

Lacetera, N., Scalia, D., Mashek, D., Bernabucci, U., and R. Grummer. 2007. Effects of

intravenous triacylglycerol emulsions on lymphocyte responses to mitogens in fasted dairy cows undergoing intense lipomobilization. J. Dair. Res., 74(3): 323-328.

Lawrence, T. 2009. The nuclear factor NF-kappaB pathway in inflammation.. Cold Spring Harb. Perspect. Biol. 1(6):1-10. doi:10.1101/cshperspect.a001651.

Lee, K. S., S. J. Park, P. H. Hwang, H. K. Yi, C. H. Song, O. H. Chai, J.-S. Kim, M. K. Lee, and Y. C. Lee. 2005. PPAR-gamma modulates allergic inflammation through up-regulation of PTEN. FASEB J. 19:1033–1035. doi:10.1096/fj.04-3309fje.

Lehnen, T. E., M. R. da Silva, A. Camacho, A. Marcadenti, and A. M. Lehnen. 2015. A review on effects of conjugated linoleic fatty acid (CLA) upon body composition and energetic metabolism. J. Int. Soc. Sports Nutr. 12(36):1-11. doi:10.1186/s12970-015-0097-4.

Lin, S., J. Hou, F. Xiang, X. Zhang, L. Che, Y. Lin, S. Xu, G. Tian, Q. Zeng, B. Yu, K. Zhang, D. Chen, D. Wu, and Z. Fang. 2013. Mammary inflammation around parturition appeared to be attenuated by consumption of fish oil rich in n-3 polyunsaturated fatty acids. Lipids Health Dis. 12(190):1-13. doi:10.1186/1476-511X-12-190.

Liu, Y., M. Biarnés Costa, and C. Gerhardinger. 2012. IL-1β Is Upregulated in the Diabetic Retina and Retinal Vessels: Cell-Specific Effect of High Glucose and IL-1β Autostimulation. PLoS One 7(5):e36949. doi:10.1371/journal.pone.0036949.

Mandard, S., and D. Patsouris. 2013. Nuclear Control of the Inflammatory Response in Mammals by Peroxisome Proliferator-Activated Receptors. PPAR Res. 2013:1–23. doi:10.1155/2013/613864.

McClenahan, D. J., J. P. Sotos, and C. J. Czuprynski. 2005. Cytokine response of bovine mammary gland epithelial cells to *Escherichia coli* , coliform culture filtrate, or lipopolysaccharide. Am. J. Vet. Res. 66:1590–1597. doi:10.2460/ajvr.2005.66.1590.

Medina-Estrada, I., J. E. López-Meza, A. Ochoa-Zarzosa, and A. Ochoa-Zarzosa. 2016. Anti-Inflammatory and Antimicrobial Effects of Estradiol in Bovine Mammary Epithelial Cells during *Staphylococcus aureus* Internalization. Mediators Inflamm. 2016:1–16. doi:10.1155/2016/6120509.

Moraes, M. L., A. M. L. Ribeiro, A. M. Kessler, V. S. Ledur, M. M. Fischer, L. Bockor, S. P. Cibulski, and D. Gava. 2012. Effect of CLA on performance and immune response of weanling piglets. J. Anim. Sci. 90:2590–2598. doi:10.2527/jas.2011-4115.

Oliveira, D. E., M. A. S. Gama, D. Fernandes, L. O. Tedeschi, and D. E. Bauman. 2012. An unprotected conjugated linoleic acid supplement decreases milk production and secretion of milk components in grazing dairy ewes. J. Dairy Sci. 95:1437–1446. doi:10.3168/jds.2011-4618.

Patterson, E., R. Wall, G. F. Fitzgerald, R. P. Ross, and C. Stanton. 2012. Health implications of high dietary omega-6 polyunsaturated Fatty acids.. J. Nutr. Metab. 2012:1-16. doi:10.1155/2012/539426.

Renner, L., S. Kersten, A. Duevel, H. J. Schuberth, and S. Dänicke. 2013. Effects of cis-9,trans-11 and trans-10, cis-12 conjugated linoleic acid, linoleic acid, phytanic acid and the combination of various fatty acids on proliferation and cytokine expression of bovine peripheral blood mononuclear cells. Nutrients. 5:2667–2683. doi:10.3390/nu5072667.

Scalia, D., N. Lacetera, U. Bernabucci, K. Demeyere, L. Duchateau, C. Burvenich, T. Aoki, T. Etoh, T. Hashimoto, M. Naruse, H. Sano, H. Utsumi, and H. Nawata. 2006. In vitro effects of nonesterified fatty acids on bovine neutrophils oxidative burst and viability. J. Dairy. Sci.. 89:147–54.

Song, H.-J., I. Grant, D. Rotondo, I. Mohede, N. Sattar, S. D. Heys, and K. W. J. Wahle. 2005. Effect of CLA supplementation on immune function in young healthy volunteers. Eur. J. Clin. Nutr. 59:508–517. doi:10.1038/sj.ejcn.1602102.

Sordillo, L. M., and W. Raphael. 2013. Significance of Metabolic Stress, Lipid Mobilization, and Inflammation on Transition Cow Disorders. Vet. Clin. North Am. Food Anim. Pract. 29:267–278. doi:10.1016/j.cvfa.2013.03.002.

Tamayo, E., A. Fernández, R. Almansa, E. Carrasco, M. Heredia, C. Lajo, L. Goncalves, J. I. Gómez-Herreras, R. O. de Lejarazu, and J. F. Bermejo-Martin. 2011. Pro- and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock. Eur. Cytokine Netw. 22:82–87. doi:10.1684/ecn.2011.0281.

Tak, P. P., and G. S. Firestein. 2001. NF-κB: a key role in inflammatory diseases. J. Clin.

Invest. 107:7–11. doi:10.1172/JCI11830.

Teng, K.-T., C.-Y. Chang, L. F. Chang, and K. Nesaretnam. 2014. Modulation of obesity-induced inflammation by dietary fats: mechanisms and clinical evidence. Nutr. J. 13(12):1-15. doi:10.1186/1475-2891-13-12.

Verlengia, R., R. Gorjão, C. C. Kanunfre, S. B. Verlengia, R., R. Gorjão, C. C. Kanunfre, S. Bordin, T. M. de Lima, E. F. Martins, P. Newsholme, and R. Curi. 2004. Effects of EPA and DHA on proliferation, cytokine production, and gene expression in Raji cells. Lipids. 39:857–864.

Zhang, J.-M., and J. An. 2007. Cytokines, inflammation, and pain. Int. Anesthesiol. Clin. 45:27–37. doi:10.1097/AIA.0b013e318034194e.

Zhang, X., R. Du, T. Zhong, W.-Q. Zhang, P. Song, W.-D. Song, Y. Zhao, C. Wang, Y.-Q. Tang, and Q. Zhang. 2014. Antitumor effect of iRGD-modified liposomes containing conjugated linoleic acid–paclitaxel (CLA-PTX) on B16-F10 melanoma. Int. J. Nanomedicine 9:3091–3105. doi:10.2147/IJN.S65664.

Zhao, G., T. D. Etherton, K. R. Martin, P. J. Gillies, S. G. West, and P. M. Kris-Etherton. 2007. Dietary alpha-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects. Am. J. Clin. Nutr. 85:385–391.

Wallace, F. A., E. A. Miles, C. Evans, T.E. Stock, P. Yaqoob, and P.C. Calder. 2001. Dietary Fatty Acids Influence the Pro-duction of Th1- but Not Th2-type Cytokines, J. Leukoc. Biol. 69:449–457.

5. General conclusions

For dairy cows, the transition period is characterized by important physiological changes that affect the health and production performance of the animals. Substantial evidence confirms that oxidative stress and inflammation during the *pre-partum* and early lactation period may contribute to several health disorders in dairy cattle. Supplementation of antioxidants and anti-inflammatory nutrients could potentially improve the health status and performance of animals. However, the therapies used until now have not achieved this consistently. Lately, scientific attention has been oriented towards the use of fatty acids such as CLA and EFAs. Moreover, a management method used in dairy cows to alleviate the magnitude of negative energy balance is feeding rumen-protected fatty acids, like CLA. The purpose of my doctoral research was to investigate and to better understand how the biologically active CLA isomers [cis-9,trans-11 and trans-10,cis-12], and EFAs [(α)-linolenic acid (aLnA), (γ)-linolenic acid (gLna) and linoleic acid (LA)] can affect the oxidative and inflammatory cellular state and so clarify their mechanism of action in bovine mammary epithelial cell culture model (BME-UV1). From this study was observed that both CLA and EFAs had antioxidant and anti-inflammatory effects. Cell exposure to 50 μM of FAs for 48 hours has strongly strengthened the cellular defenses against oxidative damage. Cells treated with fatty acids induced greater levels of reduced GSH, matched by great activity of γGCL and an increase of NADPH concentration; in particular, GSH synthesis was up-regulated by different PUFA, mainly through an induction of γGCL. Moreover, cells treated with CLA and EFAs showed a reduction in intracellular ROS and MDA levels even after H_2O_2 insult. Particularly, CLA isomers supplementation has shown a better antioxidant cellular response against oxidative damage induced by H_2O_2 compared with other FAs. All FAs reduced the gene expression of pro and anti-inflammatory cytokines, among FAs, t10,c12 CLA, LA and gLna showed an homogeneous reduction of the three pro-inflammatory cytokines and this may correspond to more balanced and efficient physiological activity and may trigger a better protective effect. The results suggest that FAs inhibited the transcription of pro-inflammatory cytokines mainly by the up-regulation of PPARγ expression.

Results from this doctoral study strengthen and corroborate the utilization of CLA and EFAs as a useful management tools for dairy cattle. In particular, it has to be considered that in the last two decades the animal husbandry is changed, and the milk yield of dairy cows is increased extremely and is still increasing. Cows receive total mixed rations based on grass

silage, corn and concentrates, but no fresh grass from pasture that provides high amounts of EFAs which are important precursors for CLA synthesis. Therefore, for the high-yielding dairy cow, the deficiency of EFAs and CLA may have an adverse effect on the oxidative and inflammatory state, productive performance and immune system. Certainly the most critical physiological condition for high-yielding dairy cow is the time of calving, when cows experience oxidative stress and are exposed to inflammation processes due to parturition. Thus, to reduce oxidative stress and the inflammatory phenomenon during transition period, it would be more appropriate to give rumen-protected EFAs and CLA.

Based on the results of the present doctoral thesis, further *in vivo* studies on EFAs and CLA supplementation on dairy cow would be necessary. These studies may provide new information on the requirement of CLA and EFAs for systemic metabolism of dairy cows, and would improve knowledge to fully understand the effects of these FAs on physiological function, on bovine mammary metabolism and to confirm the potential role of FAs in modulating the oxidative and inflammatory status of transition cows.

6. Publications

6.1 Scientific journal related to the thesis

Basiricò L, Morera P, **Dipasquale D**, Tröscher A, Bernabucci U. 2017. Comparison between CLA and essential fatty acids in preventing oxidative stress in bovine mammary epithelial cells (BME-UV1). J. Dairy Sci. 100:2299–2309.

Basiricò L, Morera P, **Dipasquale D**, Tröscher A, Serra A, Mele M, Bernabucci U. 2015. Conjugated linoleic acid isomers strongly improve the redox status of bovine mammary epithelial cells (BME-UV1) J. Dairy Sci. 98(10):7071-7082.

6.2 Other Scientific journal produced

Bernabucci U, Basiricò L, Morera P, **Dipasquale D**, Vitali A, Piccioli Cappelli F, Calamari L. 2015. Effect of summer season on milk protein fractions in Holstein cows. J. Dairy Sci. 98(3):1815-27.

6.3 Congress communications

Daniele Dipasquale, Loredana Basiricò, Patrizia Morera, Andrea Serra, Marcello Mele, Arnulf Troescher, Umberto Bernabucci. 2015. Effects of conjugated linoleic acids isomers on oxidative mammary gland metabolism. XXI *ASPA Congress* (Association for Animal Science and Productions). June 9-12, 2015, Milan, Italy. (Abstract)

Basiricò L, Troscher A, **Dipasquale D**, Morera P, Serra A, Mele M, Bernabucci U. 2015. Conjugated linoleic acid (CLA) isomers strongly improves the redox status of bovine mammary epithelial cells (BME-UV1). ADSA®-ASAS Joint Annual Meeting (JAM), July 9–12, 2015, Orlando, Florida. (Abstract)

7. Training activity

7.1 Research experience abroad

Leibniz Institute for Farm Animal Biology (FBN). Wihelm-Stahl-Allee 2, 18196 Dummerstorf (Germany) from May 11th to November 11th, 2015 (6 months). Research purpose: *Study of metabolic and oxidative effects of essential fatty acids omega 3 and omega 6, and the isomers of conjugated linoleic acid (CLA) in dairy cows.*

7.2 Participation to courses

From June 27th to July 1st, 2016. Participation at the Summer School *Milk for Cheese* 2016. Organized by ASPA (Italian Association for Animal Science and Production). Malga Juribello (TN).

From February 29th to March 4th, 2016. Participation at the course of *Experienced Beekeeper*. Organized by Agriculture Research Council-Honey bee and Silkworm Research Unit (CRA-API). Bologna.

From September 14th to September 18th, 2015. Participation at the *Course of basis statistical analysis for the livestock sciences*. Organized by ASPA, University of Pisa.

January-May, 2014: Course of *English language level B1* of the last 60 hours promoted and organized by Unit Language Services University of Tuscia, Viterbo.

January-June, 2014. Participation at *Beekeeping Course*. Organized by Department of Veterinary Prevention of Ragusa (ASP n.7). Ragusa.

7.3 Participation to conferences

November 4th 2016. Participation at the seminar *Biotechnology and nanotechnology – The future for mankind*. Dr. Octaviu Moldovan, Imedica SA. University of Tuscia.

From 19th to 20th June, 2016. Communication at the 5th Workshop: *Health and Nutrition in High-Yielding Dairy Cows*. **Daniele Dipasquale**, Loredana Basiricò, Patrizia Morera, Arnulf Troescher, Umberto Bernabucci. Comparison between CLA and other essential fatty acids in preventing oxidative stress in mammary gland. University of Wageningen, The Netherland.

December 10th, 2015. Participation at the seminar *Communication of scientific research (CRS)*. Dr. Maria Flora Mangano. University of Tuscia.

June 11th, 2015. Communication at the XXI ASPA Congress: **Daniele Dipasquale**, Loredana Basiricò, Patrizia Morera, Andrea Serra, Marcello Mele, Arnulf Troescher, Umberto Bernabucci. Effects of conjugated linoleic acids isomers on oxidative mammary gland metabolism. Italian Association for Animal Science and Productions, University of Milan.

April 15th, 2015. Participation at the congress *Sistemi di controllo predittivo multizonale delle condizioni microclimatiche degli allevamenti per il benessere dei bovini da latte*. Scientific manager Prof. U. Bernabucci. Frosinone.

April 2nd, 2014: Participation at the seminar *Quality "Phenotyping" Genetics and the Basis for some Wheat Grain Quality Traits*. Dr. Craig Morris, Director of the USDA-ARS Western Wheat Quality Laboratory (WWQL), Pullman, WA, USA. Univerity of Tuscia.

July 16th, 2014: Participation at the Workshop entitled: *Nutraceutical Product Development, Marketing, and Distribution Strategies*. University of Tuscia.

7.4 Didactics

October 20th, 2016. Speaker to seminar (2 h) *BME-UV1 cells- practical use in animal production". Related to the teaching program of Animal biotechnology*. Degree course of Agricultural and Environmental Sciences (L-25) of Dr. Loredana Basiricò. University of Tuscia.

November 16th, 2016. Speaker to seminar (2 h) *Effects of conjugated linoleic acid (CLA) isomers and essential fatty Acids acids (EFA) on oxidative and inflammatory status in bovine mammary epithelial cells (BME-UV1)*. Related to the teaching program of Quality and typical

products of the mountain areas (degree course of Mountain Sciences, L-25) of Prof. Umberto Bernabucci. University of Tuscia.

November, 2016. Tutor for the laboratory practice for students. Related to the teachings of Professor U. Bernabucci: *Animal production improvement* (Biotechnology for Agriculture, Environment and Health, LM7). University of Tuscia.

November, 2016. Tutor for the laboratory practice for students. Related to the teachings of Dr. Loredana Basiricò: *Animal biotechnology*. University of Tuscia.

8. Acknowledgements

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9. Abbreviations

AA= arachidonic acid
AKRs= aldo-keto reductase
ALDHs= aldehyde dehydrogenases
aLnA= (α)-linolenic acid
ANOVA= analysis of variance
APP= acute phase proteins
ARE= antioxidant response element
BCS= body condition score
BHB= β-hydroxy-butyrate
BME-UV1= bovine mammary epithelial cell culture model
BSA= bovine serum albumin
CAT= catalase
CD14= cluster of differentiation 14
CLA Mix= 50% c9,t11 and 50% t10,c12 CLA
CLA= conjugated linoleic acid
COX= cyclooxygenase
Cp= crossing point
DAMPs= damage-associated molecular patterns
DCFH-DA= dichloro-dihydro-fluorescein diacetate
DGLA= dihomo-γ-linolenic acid
DHA= docosahexaenoic acid
EDTA= ethylenediaminetetraacetic acid
EFAs= essential fatty acids
EPA= eicosapentaenoic acid
FAs= fatty acids
Flk-1= fetal Liver Kinase-1
GAPDH= gliceraldehyde 3-phosphate dehydrogenate
γGC= γ-glutamylcysteine
γGCL= γ-glutamylcysteine ligase
GS= glutathione synthetase
gLnA= (γ)-linolenic acid
GPx1= glutathione peroxidase
GR= glutathione reductase
GS= glutathione synthetase
GSH= glutathione
GSSG= oxidized glutathione
GST= glutathione S-transferase
IFNγ= interferon gamma
IGF-1= insulin-like growth factor
Ikβ= inhibitor of kappa β

IL-1= interleukin-1
IL-2= interleukin-2
IL-6= interleukin-6
IL-8= interleukin-8
IL-10= interleukin-10
Keap 1= kelch-like ECH-associated protein 1
LA= linoleic acid
LOOH= lipid hydroperoxides
LPS= lipopolysaccharide
LSD= least square difference
MDA= malondialdehyde
NADPH= nicotinamide adenine dinucleotide phosphate
NDA= naphthalenedicarboxyaldehyde
NEBAL= negative energy balance
NEFA= non-esterified fatty acids
NF-Kβ= nuclear factor kappa-light-chain-enhancer of activated
Nrf2= nuclear factor-erythroid 2-related factor 2
OD= optical density
PAMPs= pathogen-associated molecular patterns
PBS= phosphate buffered saline
PG= prostaglandin
PGE2= prostaglandin E2
PICs= pro-inflammatory cytokines
PLA_2= phospholipase A2
PPARα= peroxisome proliferator receptor-α
PPARγ= peroxisome proliferator receptor-γ
PRRs= pattern-recognition receptors
Prx= peroxiredoxin
PUFA= polyunsaturated fatty acids
ROS= reactive oxygen species
SEM= standard error of the means
rt-PCR= real-time PCR
sMaf= small Maf
SOD= superoxide dismutase
SSA= 5-sulfosalicylic acid
TBARS= thiobarbituric acid reactive substances
TNF-α= tumor necrosis factor-α
TLR-4= toll-like receptor-4
TrxR= thioredoxin reductase
VA= vaccenic acid
VEGF= vascular endothelial growth factor
VLDL= very-low-density lipoprotein

c9,t11= cis-9,trans-11 CLA
t10,c12= trans-10,cis-12 CLA

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Comparison between conjugated linoleic acid and essential fatty acids in preventing oxidative stress in bovine mammary epithelial cells

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ABSTRACT

Some in vitro and in vivo studies have demonstrated protective effects of conjugated linoleic acid (CLA) isomers against oxidative stress and lipid peroxidation. However, only a few and conflicting studies have been conducted showing the antioxidant potential of essential fatty acids. The objectives of the study were to compare the effects of CLA to other essential fatty acids on the thiol redox status of bovine mammary epithelia cells (BME-UV1) and their protective role against oxidative damage on the mammary gland by an in vitro study. The BME-UV1 cells were treated with complete medium containing 50 μM of *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, α-linolenic acid, γ-linolenic acid, and linoleic acid. To assess the cellular antioxidant response, glutathione, NADPH, and γ-glutamyl-cysteine ligase activity were measured 48 h after addition of fatty acids (FA). Intracellular reactive oxygen species and malondialdehyde production were also assessed in cells supplemented with FA. Reactive oxygen species production after 3 h of H_2O_2 exposure was assessed to evaluate and to compare the potential protection of different FA against H_2O_2-induced oxidative stress. All FA treatments induced an intracellular GSH increase, matched by high concentrations of NADPH and an increase of γ-glutamyl-cysteine ligase activity. Cells supplemented with FA showed a reduction in intracellular malondialdehyde levels. In particular, CLA isomers and linoleic acid supplementation showed a better antioxidant cellular response against oxidative damage induced by H_2O_2 compared with other FA.
Key words: conjugated linoleic acid, essential fatty acids, oxidative status, bovine mammary cells

INTRODUCTION

Lipids contained in dietary fat are known to be an excellent source of energy, and studies undertaken in the first quarter of the 20th century demonstrated that they were necessary for growth and normal physiological function (Spector and Kim, 2015). In recent years, interest has grown in the health properties of functional fatty acids, such as long-chain n-3 and n-6 fatty acids and CLA, because of their biological roles in cells (Bauman and Lock, 2006; Lunn and Theobald, 2006). Omega-6 and n-3 fatty acids are regarded as essential fatty acids (**EFA**) because mammals cannot synthesize them and they must be obtained from diet. Essential fatty acids form an important constituent of all cell membranes and confer to membranes properties of fluidity, thus determining and influencing the behavior of membrane-bound enzymes and receptors (Das, 2006). Among n-6 fatty acids, linoleic acid (**LnA**; 18:2n-6) is the most common n-6 PUFA, whereas α-linoleic acid (**aLnA**), belonging to *cis* n-3 PUFA, is the most prevalent n-3 fatty acid. Linoleic acid is often found in nature and is present in the seeds of most plants, except for coconut, cocoa, and palm. α-Linoleic acid, on the other hand, is found in the chloroplasts of green leafy vegetables and in the seeds of flax, rape, chia, perilla, and in walnuts (Lunn and Theobald, 2006; Simopoulos, 2008). It is from these 2 parent EFA that the n-3 and n-6 fatty acid families are derived through a series of enzyme-catalyzed desaturation and elongation reactions, which generally take place in the cell cytosol or in the mitochondria. α-Linoleic acid is metabolized to docosahexaenoic acid (22:6n-3) via eicosapentaenoic acid (**EPA**; 20:5n-3) and docosapentaenoic acid (22:5n-3), whereas LnA is metabolized to arachidonic acid (**ArA**; 20:4n-6) via γ-linolenic acid (**gLnA**; 18:3n-6) or eicosadienoic acid (20:2n-6), as 2 pathways are active (Lunn and Theobald, 2006). Arachidonic acid, the derivative of LnA, can be converted into the 2-series of thromboxanes and the 4-series of leukotrienes. These are very important, active, and short-lived hormones termed eicosanoids, which are involved in various patho-physiological processes concerning inflammatory conditions such as atherosclerosis, obesity, and inflammatory bowel disease. In contrast, the aLnA derivate, such as EPA, gives rise to an entirely different set of eicosanoids. These are the 3-series prostaglandins and

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thromboxanes and the 5-series leukotrienes, which are considered to be less inflammatory or even anti-inflammatory in comparison to the eicosanoid family derived from ArA (Patterson et al., 2012).

In dairy cows, it has been observed that dietary LnA and aLnA supplementation can be a nutritional strategy to improve reproductive performance and increase the percentage of pregnancies in lactating cows (Dirandeh et al., 2013). This is because EFA can stimulate the follicular growth and steroid production (Leroy et al., 2013). Furthermore, LnA and aLnA are able to modulate the inflammation and the immune response in dairy cows (Lessard et al., 2003; Caldari-Torres et al., 2011).

Conjugated linoleic acids are a group of constitution and conformation isomers of LnA that are formed in the course of biohydrogenation in the rumen and later endogenous conversion from vaccenic acid (Schmid et al., 2006; Bauman et al., 2008). The isomers that contain a double bond in the *trans* configuration are biologically active. The most studied bioactive CLA are the *cis*-9,*trans*-11 isomer and the *trans*-10,*cis*-12 isomer. The role of CLA on health has been investigated in animal and human studies (Pires and Grummer 2008; Mele et al., 2013). It was observed that CLA might play an important protective role in cancer, cardiovascular diseases, obesity, osteoporosis, and in immune and inflammatory responses (Benjamin and Spener, 2009; Moraes et al., 2012; Oliveira et al., 2012; Du et al., 2014).

Ruminants have evolved to feed on fresh grass and leaves. According to their feed preferences, Hofmann and Stewart (1972) classified them as concentrate selectors, intermediate-mixed feeders, or grass and roughage eaters. The dairy cow belongs to the group of grazer or roughage eaters, such as *Bos primigenius* (aurochs), relying on grasses and roughage. Modern TMR diets differ significantly from the natural diet of a cow (e.g., in its ruminal production of CLA). Elgersma et al. (2004) showed that milk from cows feeding on fresh grass quickly decreases in CLA when the diet was changed to the more modern silage or concentrate TMR type. In a review, Elgersma et al. (2006) concluded, in general, that cows on fresh grass (e.g., pasture) produce milk with a significant higher level of CLA. This observed difference in CLA content in milk triggered extensive research into increasing CLA levels in milk from TMR-fed cows, with the focus being on human health. However, these studies (Elgersma et al., 2004, 2006) triggered only limited interest considering CLA as a nutrient and the wellbeing of the cow in the first place. Studies on farm animals showed that some CLA isomers decrease milk fat synthesis, and may thus improve energy balance and alleviate energy demands in lactating dairy cattle. It has been routinely observed that CLA reduce milk fat percentage, increase milk yield, improve reproduction in early lactation, reduce incidence of metabolic disorders during early lactation (ketosis), and reduce negative effects of inflammatory processes during the periparturient period (Bauman et al., 2008; Galamb et al., 2016). Some studies have shown the protective effects of CLA isomers against oxidative stress and lipid peroxidation in animal models (Andreoli et al., 2010; Chinnadurai et al., 2013). Gessner et al. (2015) in cows, and in dairy ewes, Zeitz et al. (2015) demonstrated significantly higher vitamin E and A concentrations in milk from CLA-supplemented animals. Changes in the oxidative metabolism occur in the transition period of dairy cows, and several studies have suggested that oxidative stress increases the susceptibility of dairy cattle to diseases (Bernabucci et al., 2005; Castillo et al., 2005; Sordillo and Aitken, 2009). Nevertheless, results regarding the effects of supplementing antioxidants (vitamins and trace elements) on dairy cow health and performance have been inconsistent, because, in most cases, the antioxidant potential of the animals was not assessed beforehand and the nutritional strategies were not planned accordingly (Abuelo et al., 2015). Therefore, reviewing the redox balance in dairy cattle could help establish new nutritional strategies that could improve transition cow health.

Our recent study (Basiricò et al., 2015) showed that CLA isomers have an antioxidant role by developing a significantly high redox status in bovine mammary cells. Only a few and contradictory reports have been published regarding the protective potential of aLnA, LnA, and gLnA (Arab et al., 2006b). Fagali and Catalá (2008) demonstrated in an in vitro study that CLA exhibited greater free radical quenching activity than LnA or aLnA. Therefore, the aim of the current study was to compare the effects of CLA with other EFA on the thiol redox status of cells and their protective activity against oxidative damage on mammary gland by an in vitro model based on bovine mammary epithelial cells (BME-UV1).

MATERIALS AND METHODS

BME-UV1 Culture Conditions

The BME-UV1 cell line was created at the University of Vermont from primary bovine mammary epithelial cells in culture by stable transfection with SV40 large T-antigen; BME-UV1 cells were provided by Antonella Baldi (Department FENS, University of Milan, Italy). Cells were routinely cultivated into 75-cm^2 tissue culture flasks (Costar, Corning, NY), in a mixture of 50% DMEM-F12, 30% RPMI-1640, and 20% NCTC-135

(Sigma-Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum, 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 m*M* glutathione, 1 μg/mL of insulin, 5 μg/mL of transferrin, 1 μg/mL of hydrocortisone, 0.5 μg/mL of progesterone, 10 μg/mL of L-ascorbic acid, and antibiotics (penicillin 100 IU/mL; streptomycin 100 μg/mL). All medium supplements were from Sigma-Aldrich. The cells were maintained at 37°C in a humidified 5% CO_2 incubator. Cells used in the present work were at passage number between 39 and 41.

Experimental Design

To examine the role of fatty acids (**FA**) on metabolic and oxidative status of bovine mammary gland, BME-UV1 cells were resuspended in complete culture medium and, after 24 h of incubation, medium was removed and cells were treated for 48 h with complete medium containing 50 μ*M* *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, aLnA (18:3n-3), gLnA (18:3n-6), and LnA (18:2n-6). Control cells were not treated. Essential fatty acids were first dissolved in ethanol 95%, and the dilutions of the stock solution were made into aqueous buffers (culture medium) before performing biological experiments. To ensure that the residual amount of organic solvent was insignificant, as organic solvents may have physiological effects at low concentrations, a control test of cell viability was performed and no cytotoxic effects or biological differences were observed (data not shown).

To test the potential protection of FA against H_2O_2-induced oxidative stress, cells were treated as described followed by incubation at 37°C for 3 h with H_2O_2 (50 μ*M*); the content of reactive oxygen species (**ROS**) and malondialdehyde (**MDA**) concentration were also determined. Cell viability after 48 h from addition of FA was determined. Reactive oxygen species and MDA were also determined in cells after 48 h exposure to single FA. The fatty acids tested were purchased from Larodan (Malmo, Sweden). The experiments included at least 3 replicates per treatment and were repeated at least twice.

Cellular Antioxidant Response and Cell Protection

Cell Viability Assay. Cell viability after 48 h of incubation with FA was determined using XTT assay with Cell Proliferation kit II [XTT: sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; Roche Applied Science, Indianapolis, IL] according to the manufacturer's instructions. Briefly, cells were seeded into 96-well microplates at an optimal density (5×10^5 cells/mL) and were incubated in the same conditions described above. For each treatment, after 48 h of exposure, 50 μL of XTT labeling mixture was added to each well. After 24 h of incubation at 37°C, absorbance was measured at 450 nm.

Thiol Redox Status. The reduced (**GSH**) and the oxidized form of glutathione (**GSSG**) and the reduced form of NADPH contents as well as the activity of γ-glutamate cysteine ligase (**γGCL**) were determined for assessing the thiol redox status of BME-UV1 cells. For the determination of GSH, GSSG, and NADPH, adherent cells were detached using trypsin/EDTA solution and centrifuged at 4,500 × *g* for 5 min at 4°C. The pellet was resuspended in 200 μL of PBS and lysed by 2 cycles of sonication lasted (100 W for 30 s), centrifuged (15,000 × *g* for 5 min at 4°C), and stored at −80°C until analysis. In cell extracts, the GSH-to-GSSG ratio and NADPH concentration were determined by colorimetric assays (BioAssay Systems, Hayward, CA). Optical density was measured by a spectrophotometer at 405 and 540 nm for GSH/GSSG and NADPH, respectively.

The γGCL activity was determined by a fluorescence assay, as described by Chen et al. (2010). The BME-UV1 cells were detached using trypsin/EDTA solution and centrifuged at 4,500 × *g* for 5 min at 4°C. The pellet was resuspended in 100 μL of TES/SB buffer (wt/vol, 1/4) consisting of 20 m*M* Tris, 1 m*M* EDTA, 250 m*M* sucrose, 20 m*M* sodium borate, and 2 m*M* serine. The cells were sonicated at 100 W for 60 s and then centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatants were collected and centrifuged again at 15,000 × *g* at 4°C for 20 min. The supernatants were collected and the protein concentrations were determined using a BCA Protein Assay Kit from Pierce (Rockford, IL), with BSA used as the standard. For the γGCL activity assay, aliquots of 30 μL of supernatant were mixed with 30 μL of γGCL reaction cocktail (400 m*M* Tris, 40 m*M* ATP, 40 m*M* L-glutamic acid, 2 m*M* EDTA, 20 m*M* sodium borate, 2 m*M* serine, and 40 m*M* $MgCl_2$). Following incubation at 37°C for 5 min, 30 μL of cysteine solution (30 m*M*; dissolved in TES/SB buffer) was added and the mixtures were incubated at 37°C for 13 min. The enzymatic reaction in the mixture was stopped by precipitating proteins with 200 m*M* 5-sulfosalicylic acid. After placing on ice for 20 min, the mixtures were centrifuged at 2,000 × *g* at 4°C for 10 min. Following centrifugation, 20 μL of each supernatant containing γ-glutamylcysteine (**γGC**) was added to a 96-well plate designed for fluorescence detection. For each assay, 20 μL of γGC standards, containing 5 μL of γGC reaction cocktail [5 μL of 5-sulfosalicylic acid (200 m*M*), 5 μL of H_2O, and 5 μL of γGC standard solution (0, 20, 40, 60, 80, 100, 120, and 140 μ*M* in TES/SB buffer)] was added to each well of the same 96-well plate to generate a standard curve. Next, 180

μL of 2,3-naphthalenedicarboxyaldehyde was added to each well. Following incubation in the dark at room temperature for 30 min, the formation of 2,3-naphthalenedicarboxyaldehyde–γGC was measured (472 nm excitation/528 nm emission) using a fluorescent plate reader (Multimode Detector DTX 880, Beckman Coulter Inc., Fullerton, CA). The production of γGC in each sample was calculated using the standard curve. Values were expressed in micromoles per minute per microgram of total proteins.

Measurement of MDA Production. For the determination of MDA, adherent cells were detached using trypsin/EDTA solution and centrifuged at 4,500 × *g* for 5 min at 4°C. The pellet was resuspended in 200 μL of PBS and lysed by 2 cycles of sonication at 100 W for 30 s, centrifuged at 15,000 × *g* for 5 min at 4°C, and stored at −80°C until analysis. In cell extracts MDA concentrations were determined by colorimetric assays (Abcam, Cambridge, UK). Optical density was measured by a spectrophotometer at 540 nm.

Measurement of ROS Production. To determine ROS concentration, cells were washed twice with PBS and incubated with 20 μ*M* 2′,7′-dichlorodihydrofluorescin diacetate probe (DCFH-DA) in PBS at 37°C for 40 min. Fluorescence was measured at 485 (excitation) and 535 nm (emission) wavelengths on a microplate reader (Multimode Detector DTX 880, Beckman Coulter Inc.).

Statistical Analysis

All data of the experiment are presented as least squares means and standard error of the means. The data were analyzed by ANOVA using Statistica-7 software package (StatSoft Inc., Tulsa, OK). The significance of the differences was assessed by the Fisher's least square difference (LSD) test. Significance was declared at $P < 0.05$.

RESULTS

Effect of FA on Cell Viability in BME-UV1

Cell viability was carried out using the XTT assay and was evaluated after 48 h of FA exposure. As shown in Figure 1, no FA treatment reduced cell viability, and no differences were observed between treatments. This assay indicated that the exposure to different FA did not show any cytotoxic effect.

Effect of FA on Thiol Redox Status in BME-UV1

Oxidized and Reduced Glutathione. The levels of nonenzymatic antioxidants, such as GSH, are depicted in Figure 2. Compared with the control, an increase ($P < 0.01$) of reduced GSH was showed in cells treated with *cis*-9,*trans*-11 CLA, aLnA, gLnA, LnA, and with

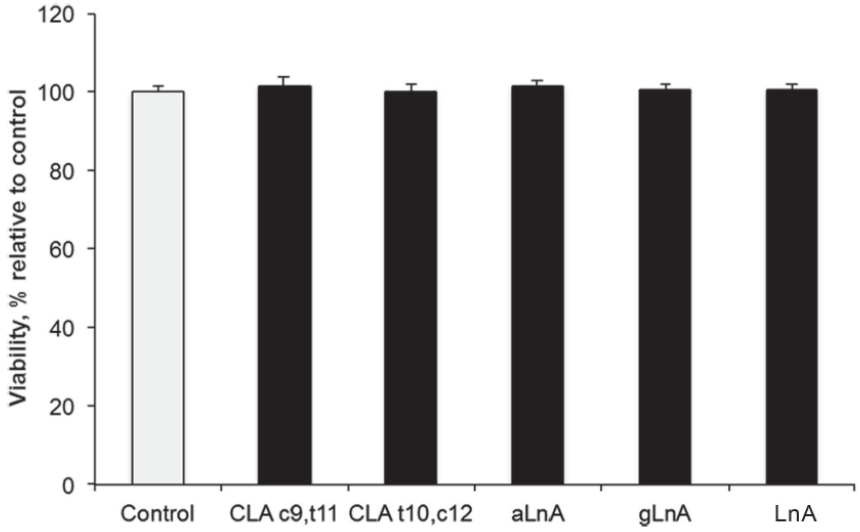

Figure 1. Cell viability of bovine mammary epithelia cells (BME-UV1) cells evaluated after 48 h of exposure to fatty acids. Data are reported as LSM ± SEM (n = 6). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

trans-10,*cis*-12 CLA ($P < 0.05$; Figure 2A). Among treatments, values of reduced GSH were significantly higher ($P < 0.01$) in cells treated with aLnA and LnA compared with *cis*-9,*trans*-11 CLA, gLnA, and *trans*-10,*cis*-12 CLA. Reduced GSH levels in cells treated with *cis*-9,*trans*-11 CLA and gLnA were not different, but differed significantly ($P < 0.01$) from those treated with *trans*-10,*cis*-12 CLA. No differences in the level of oxidized GSH were noted for cells treated with FA compared with the control culture (Figure 2B). The ratio of GSH to GSSG showed the same trend of reduced GSH (Figure 2C).

Quantitative Determination of NADPH. Concentrations of a redox coenzyme NADPH are reported in Figure 3. Compared with the control, a greater level of NADPH was observed in cells treated with *cis*-9,*trans*-11 CLA, aLnA, gLnA, LnA ($P < 0.01$), and *trans*-10,*cis*-12 CLA ($P < 0.05$). The *cis*-9,*trans*-11 CLA showed greater ($P < 0.01$) concentration of NADPH than all other FA; the values of NADPH were not significantly different between aLnA, gLnA, and LnA, whereas NADPH in the *trans*-10,*cis*-12 CLA-treated cells was significantly lower ($P < 0.05$) than cells treated with aLnA and LnA.

γ-GC Ligase Activity. Thiol redox status was also assessed by measuring γGCL activity (Figure 4). After the 48 h, all FA induced a strong antioxidant response by increasing ($P < 0.01$) the intracellular γGCL activity compared with the control. Among treatments, higher γGCL activity ($P < 0.01$) was observed in gLnA treatment compared with other FA. The enzyme activity was not different between *cis*-9,*trans*-11 CLA, aLnA, and LnA treatments, and was lower ($P < 0.01$) in cells treated with *trans*-10,*cis*-12 CLA compared with other FA.

Intracellular ROS and MDA. Intracellular ROS production by dichlorofluorescein (DCFH-DA) fluorescence measurement was assessed in cells supplemented with FA (Figure 5A). Cells treated with FA showed an increase ($P < 0.01$) of ROS production compared with the control. Cells treated with gLnA showed the greatest levels ($P < 0.01$) of ROS compared with the other FA. Both CLA isomers showed the same and lower levels ($P < 0.01$) of ROS production compared with other FA treatments.

Cell concentrations of MDA are shown in Figure 5B. All FA reduced ($P < 0.01$) the MDA level compared with untreated cells. Among treatments, cells treated with *trans*-10,*cis*-12 CLA and LnA showed similar MDA levels and much more lower (*P*-value ranged from <0.05 to <0.01) than the other FA. Malondialdehyde in cells treated with aLnA was not different than *trans*-10,*cis*-12 CLA, but was greater ($P < 0.01$) compared with LnA. The MDA values of *cis*-9,*trans*-11 CLA was greater ($P < 0.01$) than *trans*-10,*cis*-12 CLA, aLnA, and LnA. Cells treated with gLnA showed greater ($P < 0.01$) levels of MDA compared with other FA.

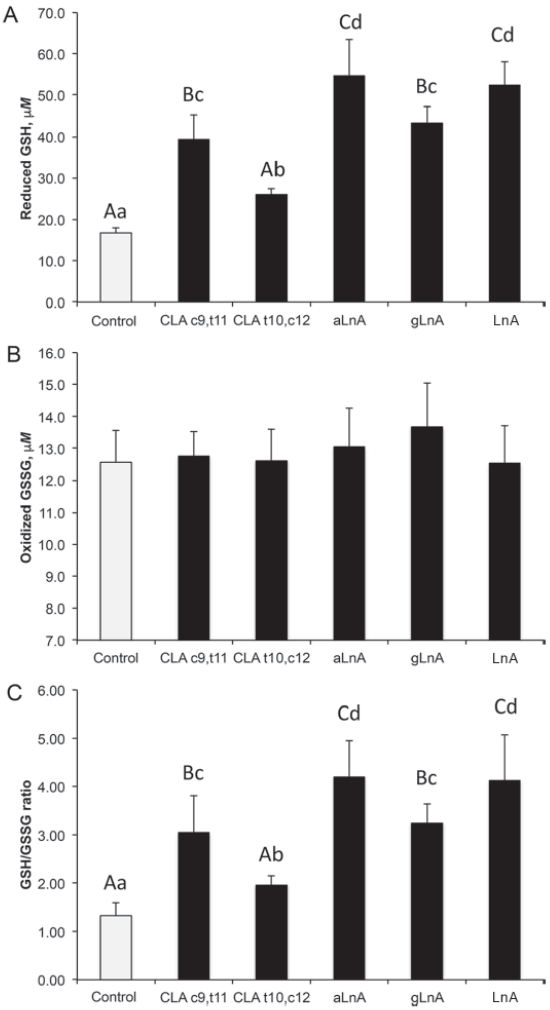

Figure 2. Intracellular concentration of reduced glutathione (GSH; A), oxidized glutathione (GSSG; B), and GSH-to-GSSG ratio (C) in bovine mammary epithelia cells (BME-UV1) after 48 h of exposure to fatty acids. Data are reported as least squares means ± SEM (n = 6). Significant differences among control and treatments are represented by different letters (a–d = $P < 0.05$; A–C = $P < 0.01$). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

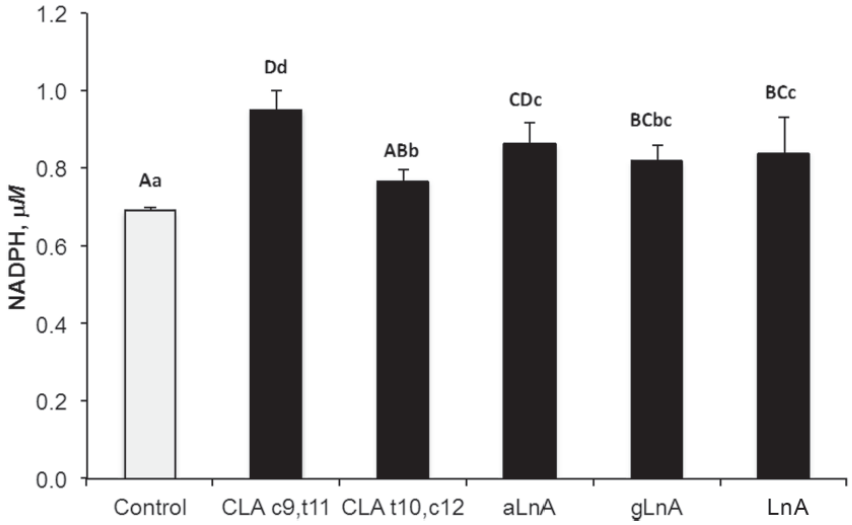

Figure 3. Intracellular concentration of NADPH after 48 h of exposure to fatty acids. Data are reported as least squares means ± SEM (n = 6). Significant differences among control and treatments are represented by different letters (a–d = $P < 0.05$; A–D = $P < 0.01$). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

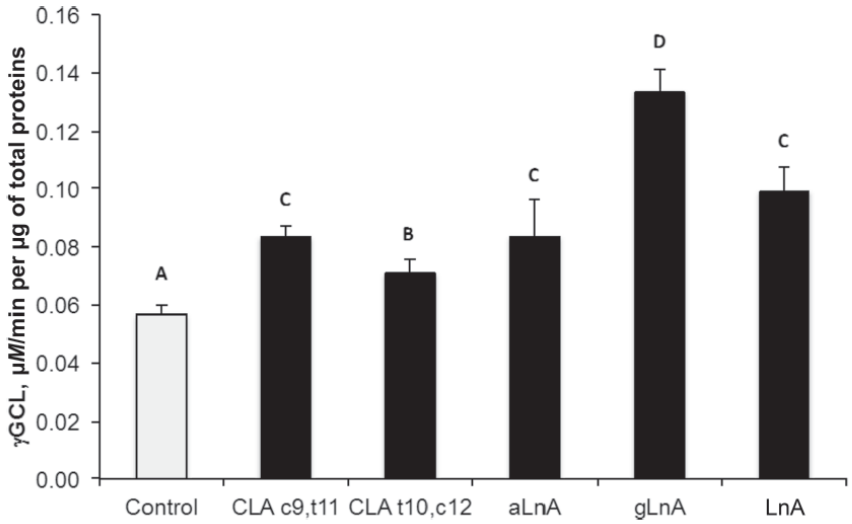

Figure 4. Intracellular activity of γ-glutamate cysteine ligase (γGCL) after 48 h of exposure to fatty acids. Data are reported as least squares means ± SEM (n = 6). Significant differences among control and treatments are represented by different letters (A–D = $P < 0.01$). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

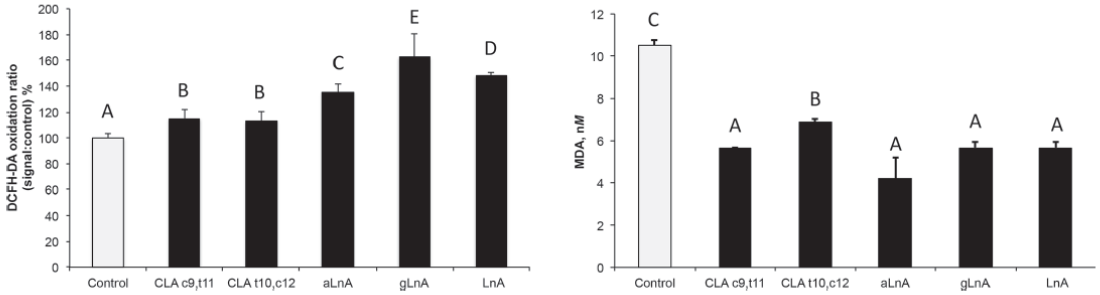

Figure 5. Intracellular production of reacting oxygen species by dichloro-dihydro-fluorescein diacetate assay (DCFH-DA) and malondialdehyde (MDA; B) in bovine mammary epithelia cells (BME-UV1) cells after 48 h of exposure to fatty acids. Data are reported as least squares means ± SEM (n = 6). Significant differences among control and treatments are represented by different letters (A–E = $P < 0.01$). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

Effect of FA on Protection of Cells Against Oxidative Stress

To evaluate and to compare the potential protection of different FA against H_2O_2-induced oxidative stress, ROS test after 3 h to H_2O_2 exposure was assessed. As shown in Figure 6, all FA were not able to enhance cell resistance against oxidative stress at 48 h. The CLA-treated cells showed a ROS production similar to the control and significantly lower ($P < 0.01$) than the other FA. The ROS production was greater ($P < 0.01$) in cells treated with, aLnA, gLnA, and LnA compared with the control. Cells treated with gLnA showed a greater ($P < 0.01$) concentration of ROS compared with the other FA. All FA showed lower ($P < 0.01$) MDA levels compared with control cells (Figure 7). Among FA, aLnA and gLnA showed higher ($P < 0.05$) MDA levels compared with their counterparts.

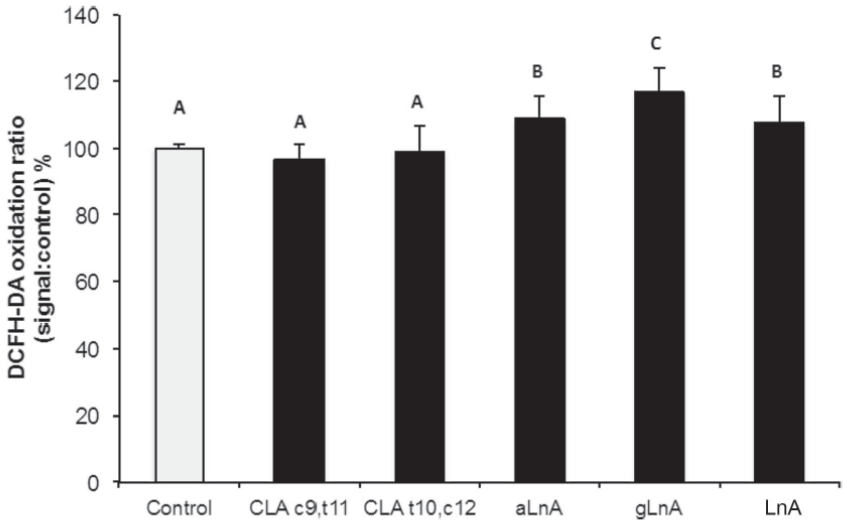

Figure 6. Intracellular production of reacting oxygen species by dichloro-dihydro-fluorescein diacetate assay (DCFH-DA). After 48 h of exposure to fatty acids, cells were treated for 3 h with H_2O_2 (50 μ*M*). Data are reported as least squares means ± SEM (n = 6). Significant differences among control and treatments are represented by different letters (A–C = $P < 0.01$). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

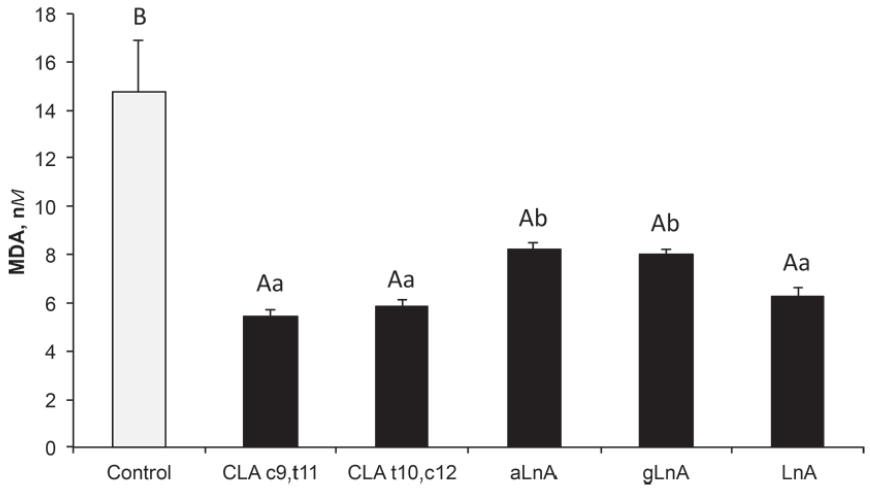

Figure 7. Intracellular production of malondialdehyde (MDA). After 48 h of exposure to fatty acids, cells were treated for 3 h with H_2O_2 (50 μM). Data are reported as least squares means ± SEM (n = 6). Significant differences among control and treatments are represented by different letters (a,b = $P < 0.05$; A,B = $P < 0.01$). c = *cis*; t = *trans*; aLnA = α-linolenic acid; gLnA = γ-linolenic acid; LnA = linoleic acid.

DISCUSSION

In the present report we investigated the effect of several FA (*cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, aLnA, gLnA, and LnA) on the cellular antioxidant response and on lipoperoxidation in BME-UV1 cells. Our results have shown that the bovine cell line BME-UV1 has different susceptibilities to treatment with different EFA and CLA.

In our experimental conditions, the exposure to 50 μM FA for 48 h strengthened the cellular defenses against to oxidative damage compared with control cells. We observed that the cell supplementation of FA induced higher levels of reduced GSH, matched by high activity of γGCL and an increase of NADPH concentration; in particular, GSH synthesis was upregulated by different PUFA, mainly through an induction of γGCL. The data presented here are consistent with our previous study (Basiricò et al., 2015) and with other findings indicating an improved antioxidant status in cells treated with CLA and EFA (Arab et al., 2006b; Espinosa-Diez et al., 2015).

Reduced glutathione plays a primary role in the maintenance of intracellular redox homeostasis by affording protection against reactive oxygen and nitrose species as well as electrophilic xenobiotics. Under normal cellular redox conditions, the major portion of this redox regulator is in the form of GSH, whereas GSSG concentration increases during oxidative stress. Reduced glutathione is synthesized from its constituent AA, forming a tripeptide thiol; this synthesis requires 2 ATP-dependent steps. The first and limiting synthesis is catalyzed by γGCL and the second step is mediated by GSH synthetase. Above all, an induction of γGCL is characteristic of an antioxidant response (Lee et al., 2003; Rahman, 2005). Moreover, NADPH is essential in GSH recycling and related antioxidant functions, and the ratio of the reduced to total pyridine nucleotide pool is generally accepted as an indicator of the cellular redox status (Adams et al., 2001).

In vivo study on rats fed oils with high levels of aLnA improved the antioxidant status of hepatic tissue with the increase of GSH content and the GSH-to-GSSG ratio without modification of the levels of GSSG (Rincón-Cervera et al., 2016). Ramos and Colquhoun (2003) observed that gLnA and EPA in rat C6 glioma cell increased NADPH production, permitting the maintenance of adequate intracellular reduced glutathione concentrations and limiting rates of lipid peroxidation and ROS generation. Bergamo et al. (2011) reported that isomeric mixture of CLA (*cis*-9,*trans*-11 and *trans*-10,*cis*-12) administration in MGFD mice resulted in a significant enhancement of GSH content and upregulated the expression of the γGCL mRNA and protein. The upregulation of γGCL and glutathione reductase was observed in human fibroblast cells treated for 24 and 48 h with docosahexaenoic acid (n-3 PUFA; Arab et al., 2006a). Mollica et al. (2014) showed that *cis*-9,*trans*-11 CLA supplementation to rats improves the GSH-to-GSSG ratio in the liver, and nuclear factor

erythroid 2-related factor 2 (**Nrf2**) activity. Cui et al. (2016), in primary mouse hepatocytes treated with different FA (saturated and unsaturated, including LnA, aLnA, and AA) for 24 h, observed an enhancement of Nrf2 transcription factor at the mRNA and protein levels. Bergamo et al. (2008), in murine dendritic cells, demonstrated the ability of *cis*-9,*trans*-11 CLA to activate cytoprotective mechanisms induced via Nrf2/ARE (antioxidant response element).

These results provide a basis for the hypothesis that, in our experiments, the capacity of FA to positively stimulate the availability of antioxidant enzyme could be related to the ability in activating the Nrf2 transcription factor. The Nrf2 pathway is the major regulator of cytoprotective responses to oxidative and electrophilic stress (Abuelo et al., 2015; Espinosa-Diez et al., 2015). Under basal conditions, Nrf2 is sequestered in the cytosol by a Keap1 homodimer, which facilitates the ubiquitination and proteasomal degradation of Nrf2. Inducers react with specific cysteine residues in Keap1, leading to the release of Nrf2, owing its nuclear translocation. In the nucleus, Nrf2 heterodimerizes together with small Maf proteins and binds to the antioxidant response element, activating the expression of a battery of cytoprotective genes, such as NAD(P)H:quinone oxidoreductase (NQO1), glutathione S-transferases, and γGCL (Lee et al., 2003).

On BME-UV1 cells, in addition to the evaluation of antioxidants, ROS amount and MDA concentration were measured. Although the amount of free radicals was higher in all cells treated with FA compared with the control, the cells treated with *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA showed lower levels of ROS production than the other FA. Espinosa-Diez et al. (2015) reported that low or moderate concentrations of ROS, historically considered as cellular damaging agents, are also involved in physiological responses as part of signaling processes, such as activation or deactivation cycles of redox metabolism and expression of antioxidant enzymes expression that are essential to maintain oxidant-antioxidant balance in the cells. In fact, ROS protects the cell against ROS damage by inducing different antioxidant responses and re-establishing or maintaining redox homeostasis. Moreover, ROS can activate Nrf2 (Nair et al., 2008), which in turn counteracts proinflammatory signaling pathways (Kim et al., 2010). These results support the hypothesis that the treatment of BME-UV1 with FA might initiate cellular antioxidant responses through Nrf2-mediated activation of antioxidant and detoxifying enzymes.

Malondialdehyde concentration has been employed for some time as a biomarker for lipid peroxidation of n-3 and n-6 FA (Ayala et al., 2014). Lipid peroxidation can be described generally as a process under which oxidants, such as free radicals, take electrons from lipids. Lipid peroxidation is a chain reaction initiated by the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of PUFA (Repetto et al., 2012). In our study, all FA supplementation decreased the cellular concentration of MDA compared with the control. This would demonstrate a protective effect of FA against the lipoperoxidation. In fact, the lowest presence of end-products such as MDA can be translated into a lower concentration of peroxyl radical and hydroperoxyl, both triggering factors of lipid oxidation. The reduced concentration of oxidizing agents may be linked to the enhanced GSH content and rely on γGCL and NADPH induction.

Finally, the measurement of ROS and MDA production in the presence of H_2O_2 were used as further tests to check and compare the potential protection of different FA against H_2O_2-induced oxidative stress. The protective effects of the 5 FA studied were not identical in mammary cells, and FA induced different ROS and MDA levels in bovine mammary epithelial cells. Hydrogen peroxide challenge of cells, which were preincubated with LnA, aLnAn or gLnAn produced oxidative damage beyond the control group whereas the same challenge of the cells with CLA isomers did not differ from the control cells in terms of oxidative stress. This might also be explained by extracellular formed lipid(per)oxidation products.

All FA strongly reduced the lipoperoxidation in cells after H_2O_2 exposure. Both CLA and LA seem to have a better protective effect compared with aLnA and gLnA. The antioxidative effect of CLA against lipoperoxidation has been recently demonstrated in lactating dairy cows (Hanschke et al., 2016).

This is an interesting finding, considering the fact that in the course of evolution cows developed the ability to alter EFA through biohydrogenation, leading to the synthesis of CLA, which may play a more significant role than the EFA ingested from dietary sources. Conjugated linoleic acid protection seems to be mediated not only to the levels of GSH, as reduced GSH and GSH-to-GSSG ratio and the activity of γGCL enzyme were lower compared with other FA. In addition, Arab et al. (2006b) reported that, among the 8 FA tested, CLA was the only PUFA able to have a protective action against oxidative insult in human skin fibroblasts. Richard et al. (2008) reported that supplementation of human aortic endothelial cells with PUFA of the n-3 series resulted in lower formation of ROS as compared with cells supplemented with SFA, MUFA, or PUFA of the n-6 series. Furthermore, because of varying protective mechanisms by different FA on intracellular oxidative stress and cell response, the expressions of ROS production, with or without H_2O_2, did not show the

same variation tendency for all FA. Interestingly, the results indicated that, in BME-UV1 cells, gLnA was more potent in inducing ROS production and MDA than other UFA. A possible explanation could be that gLnA, being a precursor of ArA, can be quickly used as inflammatory mediator.

The capability to mitigate the negative effects of H_2O_2 by CLA isomers better compared with other FA can be explained, in part, by the fact that lactating cows have the ability to endogenously synthesize CLA. In particular, the mammary gland plays a role of primary importance in the endogenous synthesis of *cis*-9,*trans*-11 CLA. Differences in cellular uptake, incorporation, and metabolization of CLA compared with EFA probably play a significant role in the effect of these different compounds on cell response. Given the results of this and of other studies, we conclude that CLA isomers are more effective on cellular level than EFA because they are natural and more familiar to ruminant cells.

CONCLUSIONS

We investigated the effect of several FA on the cellular antioxidant response and on lipoperoxidation in BME-UV1 cells. Results indicate that all FA were able to upregulate the γ-GCL activity and enhance GSH synthesis and reduce lipoperoxidation. It is worth noting that CLA isomers supplementation, compared with other EFA, does not contribute to further increase of H_2O_2-induced ROS production. The increase of GSH synthesis observed in cells treated with all FA is not sufficient to control H_2O_2 production induced by H_2O_2. In this study, the increased availability of GSH was probably used by other pathways, including reactions of transferases with xenobiotics or with lipid peroxidation products. As ruminants have a natural supply of CLA, further research should consider whether this feature is an evolutionary advantage for the cow.

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REFERENCES

Abuelo, A., J. Hernandez, J. L. Benedito, and C. Castillo. 2015. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. J. Anim. Physiol. Anim. Nutr. (Berl.) 99:1003–1016.

Adams, J. D., Jr., L. K. Klaidman, M. L. Chang, and J. Yang. 2001. Brain oxidative stress—Analytical chemistry and thermodynamics of glutathione and NADPH. Curr. Top. Med. Chem. 1:473–482.

Andreoli, M. F., P. G. Illesca, M. A. González, and C. A. Bernal. 2010. Conjugated linoleic acid reduces hepatic steatosis and restores liver triacylglycerol secretion and the fatty acid profile during protein repletion in rats. Lipids 45:1035–1045.

Arab, K., A. Rossary, F. Flourie, Y. Tourneur, and J. P. Steghens. 2006a. Docosahexaenoic acid enhances the antioxidant response of human fibroblasts by upregulating γ-glutamyl-cysteinyl ligase and glutathione reductase. Br. J. Nutr. 95:18–26.

Arab, K., A. Rossary, L. Soulère, and J. P. Steghens. 2006b. Conjugated linoleic acid, unlike other unsaturated fatty acids, strongly induces glutathione synthesis without any lipoperoxidation. Br. J. Nutr. 96:811–819.

Ayala, A., M. F. Muñoz, and S. Argüelles. 2014. Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid. Med. Cell. Longev. 2014:360438.

Basiricò, L., P. Morera, D. Dipasquale, A. Tröscher, A. Serra, M. Mele, and U. Bernabucci. 2015. Conjugated linoleic acid isomers strongly improve the redox status of bovine mammary epithelial cells (BME-UV1). J. Dairy Sci. 98:7071–7082.

Bauman, D. E., and A. L. Lock. 2006. Conjugated linoleic acid: Biosynthesis and nutritional significance. Pages 93–136 in Advances in Conjugated Linoleic Acid Research, Vol. 2, 3rd ed. AOCS Press, Springer, New York, NY.

Bauman, D. E., J. W. I. Perfield, K. J. Harvatine, and L. H. Baumgard. 2008. Regulation of fat synthesis by conjugated linoleic acid: lactation and the ruminant model. J. Nutr. 138:403–409.

Benjamin, S., and F. Spener. 2009. Conjugated linoleic acids as functional food: An insight into their health benefits. Nutr. Metab. (Lond.) 6:36.

Bergamo, P., M. Gogliettino, G. Palmieri, E. Cocca, F. Maurano, R. Stefanile, M. Balestrieri, G. Mazzarella, C. David, and M. Rossi. 2011. Conjugated linoleic acid protects against gliadin-induced depletion of intestinal defenses. Mol. Nutr. Food Res. 55:S248–256.

Bergamo, P., F. Maurano, R. D'Arienzo, C. David, and M. Rossi. 2008. Association between activation of phase 2 enzymes and down-regulation of dendritic cell maturation by c9,t11-conjugated linoleic acid. Immunol. Lett. 117:181–190.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J. Dairy Sci. 88:2017–2026.

Caldari-Torres, C., A. L. Lock, C. R. Staples, and L. Badinga. 2011. Performance, metabolic, and endocrine responses of periparturient Holstein cows fed 3 sources of fat. J. Dairy Sci. 94:1500–1510.

Castillo, C., J. Hernandez, A. Bravo, M. Lopez-Alonso, V. Pereira, and J. L. Benedito. 2005. Oxidative status during late pregnancy and early lactation in dairy cows. Vet. J. 169:286–292.

Chen, C. N., H. M. Brown-Borg, S. G. Rakoczy, D. A. Ferrington, and L. V. Thomposon. 2010. Aging impairs the expression of the catalytic subunit of glutamate-cysteine ligase in soleus muscle under stress. J. Gerontol. A Biol. Sci. Med. Sci. 65:129–137.

Chinnadurai, K., H. K. Kanwal, A. K. Tyagi, C. Stanton, and P. Ross. 2013. High conjugated linoleic acid enriched ghee (clarified butter) increases the antioxidant and antiatherogenic potency in female Wistar rats. Lipids Health Dis. 12:121.

Cui, Y., W. Qiuju, T. Xing, and Z. Xiuying. 2016. Effects of fatty acids on CYP2A5 and Nrf2 expression in mouse primary hepatocytes. Biochem. Genet. 54:29–40.

Das, U. N. 2006. Biological significance of essential fatty acids. J. Assoc. Physicians India 54:309–319.

Dirandeh, E., A. Towhidi, S. Zeinoaldini, M. Ganjkhanlou, Z. Ansari Pirsaraei, and A. Fouladi Nashta. 2013. Effects of different polyunsaturated fatty acid supplementations during the postpartum periods of early lactating dairy cows on milk yield, metabolic responses, and reproductive performances. J. Anim. Sci. 91:713–721.

Du, R., T. Zhong, W. Q. Zhang, P. Song, W. D. Song, Y. Zhao, C. Wang, Y. Q. Tang, X. Zhang, and Q. Zhang. 2014. Antitumor effect of iRGD-modified liposomes containing conjugated linoleic acid-paclitaxel (CLA-PTX) on B16–F10 melanoma. Int. J. Nanomedicine 9:3091–3105.

Elgersma, A., G. Ellen, H. van der Horst, H. Boer, P. R. Dekker, and S. Tamminga. 2004. Quick changes in milk fat composition from

cows after transition from fresh grass to a silage diet. Anim. Feed Sci. Technol. 117:13–27.
Elgersma, A., S. Tamminga, and G. Ellen. 2006. Modifying milk composition through forage. Anim. Feed Sci. Technol. 131:207–225.
Espinosa-Diez, C., V. Miguel, D. Mennerich, T. Kietzmann, P. Sánchez-Pérez, S. Cadenas, and S. Lamas. 2015. Antioxidant responses and cellular adjustments to oxidative stress. Redox Biol. 6:183–197.
Fagali, N., and A. Catalá. 2008. Antioxidant activity of conjugated linoleic acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH techniques. Biophys. Chem. 137:56–62.
Galamb, E., V. Faigl, M. Keresztes, Z. Csillik, A. Tröscher, P. Elek, M. Kulcsár, G. Huszenicza, H. Fébel, and F. Husvéth. 2016. Effect of pre- and post-partum supplementation with lipid-encapsulated conjugated linoleic acid on milk yield and metabolic status in multiparous high-producing dairy cows. J. Anim. Physiol. Anim. Nutr. (Berl.) https://doi.org/10.1111/jpn.12544.
Gessner, D. K., E. Most, G. Schlegel, K. Kupczyk, F. J. Schwarz, and K. Eder. 2015. Concentrations of retinol and tocopherols in the milk of cows supplemented with conjugated linoleic acid. J. Anim. Physiol. Anim. Nutr. (Berl.) 99:1039–1046.
Hanschke, N., M. Kankofer, L. Ruda, M. Holtershinken, U. Meyer, J. Frank, S. Danicke, and J. Rehage. 2016. The effect of conjugated linoleic acid supplements on oxidative and antioxidative status of dairy cows. J. Dairy Sci. 99:8090–8102.
Hofmann, R. R., and D. R. M. Stewart. 1972. Grazer or Browser: A classification based on the stomach structure and feeding habits of East African ruminants. Marnmalia 36:226–240.
Kim, Y. R., J. E. Oh, M. S. Kim, M. R. Kang, S. W. Park, J. Y. Han, H. S. Eom, N. J. Yoo, and S. H. Lee. 2010. Oncogenic Nrf2 mutations in squamous cell carcinomas of oesophagus and skin. J. Pathol. 220:446–451.
Lee, J. M., M. J. Calkins, K. Chan, Y. W. Kan, and J. A. Johnson. 2003. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. J. Biol. Chem. 278:12029–12038.
Leroy, J. L. M. R., R. G. Sturmey, V. Van Hoeck, J. De Bie, P. J. McKeegan, and P. E. J. Bols. 2013. Dietary lipid supplementation on cow reproductive performance and oocyte and embryo viability: A real benefit? Anim. Reprod. 10:258–267.
Lessard, M., N. Gagnon, and H. V. Petit. 2003. Immune response of postpartum dairy cows fed flaxseed. J. Dairy Sci. 86:2647–2657.
Lunn, J., and H. E. Theobald. 2006. The health effects of dietary unsaturated fatty acids. Nutr. Bull. 31:178–224. https://doi.org/10.1111/j.1467-3010.2006.00571.x.
Mele, M. C., G. Cannelli, G. Carta, L. Cordeddu, M. P. Melis, E. Murru, C. Stanton, and S. Banni. 2013. Metabolism of c9,t11-conjugated linoleic acid (CLA) in humans. Prostaglandins Leukot. Essent. Fatty Acids 89:115–119.
Mollica, M. P., G. Trinchese, G. Cavaliere, C. De Filippo, E. Cocca, M. Gaita, A. Della-Gatta, A. Marano, G. Mazzarella, and P. Bergamo. 2014. c9,t11-conjugated linoleic acid ameliorates steatosis by modulating mitochondrial uncoupling and Nrf2 pathway. J. Lipid Res. 55:837–849.
Moraes, M. L., A. M. L. Ribeiro, A. M. Kessler, V. S. Ledur, M. M. Fischer, L. Bockor, and D. Gava. 2012. Effect of CLA on performance and immune response of weanling piglets. J. Anim. Sci. 90:2590–2598.
Nair, S., S. T. Doh, J. Y. Chan, A. N. Kong, and L. Cai. 2008. Regulatory potential for concerted modulation of Nrf2- and Nfkb1-mediated gene expression in inflammation and carcinogenesis. Br. J. Cancer 99:2070–2082.
Oliveira, D. E., M. A. S. Gama, D. Fernandes, L. O. Tedeschi, and D. E. Bauman. 2012. An unprotected conjugated linoleic acid supplement decreases milk production and secretion of milk components in grazing dairy ewes. J. Dairy Sci. 95:1437–1446.
Patterson, E., R. Wall, G. F. Fitzgerald, R. P. Ross, and C. Stanton. 2012. Health implications of high dietary omega-6 polyunsaturated fatty acids. J. Nutr. Metab. 2012:539426.
Pires, J. A. A., and R. R. Grummer. 2008. Specific fatty acids as metabolic modulators in the dairy cow. Rev. Bras. Zootec. 37:287–298.
Rahman, I. 2005. Regulation of glutathione in inflammation and chronic lung diseases. Mutat. Res. 579:58–80.
Ramos, K. L., and A. Colquhoun. 2003. Protective role of glucose-6-phosphate dehydrogenase activity in the metabolic response of C6 rat glioma cells to polyunsaturated fatty acid exposure. Glia 43:149–166.
Repetto, M., J. Semprine, and A. Boveris. 2012. Lipid peroxidation: Chemical mechanism, biological implications and analytical determination. Chapter 1 in Lipid Peroxidation, A. Catala (Ed.), InTech, Rijeka, Croatia. https://doi.org/10.5772/45943.
Richard, D., K. Kefi, U. Barbe, P. Bausero, and F. Visioli. 2008. Polyunsaturated fatty acids as antioxidanta. Pharmacol. Res. 57:451–455.
Rincón-Cervera, M. Á., R. Valenzuela, M. C. Hernandez-Rodas, C. Barrera, A. Espinosa, M. M. Arambio, and A. Valenzuela. 2016. Vegetable oils rich in alpha linolenic acid increment hepatic n-3 LCPUFA, modulating the fatty acid metabolism and antioxidant response in rats. Prostaglandins Leukot. Essent. Fatty Acids https://doi.org/10.1016/j.plefa.2016.02.002.
Schmid, A., M. Collomb, R. Sieber, and G. Bee. 2006. Conjugated linoleic acid in meat and meat products: A review. Meat Sci. 73:29–41.
Simopoulos, A. P. 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp. Biol. Med. (Maywood) 233:674–688.
Sordillo, L. M., and S. L. Aitken. 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet. Immunol. Immunopathol. 128:104–109.
Spector, A. A., and H. Y. Kim. 2015. Discovery of essential fatty acids. J. Lipid Res. 56:11–21.
Zeitz, J. O., E. Most, and K. Eder. 2015. Effect of conjugated linoleic acid on concentrations of fat-soluble vitamins in milk of lactating ewes. J. Dairy Sci. 98:7328–7334.

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Conjugated linoleic acid isomers strongly improve the redox status of bovine mammary epithelial cells (BME-UV1)

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ABSTRACT

Some studies have shown the protective effects of conjugated linoleic acid (CLA) isomers against oxidative stress and lipid peroxidation in animal models, but no information is available about CLA and changes in oxidative status of the bovine mammary gland. The objectives of the study were to assess in vitro the effect of CLA on the cellular antioxidant response of bovine mammary cells, to examine whether CLA isomers could play a role in cell protection against the oxidative stress, and to study the molecular mechanism involved. For the study, BME-UV1 cells, a bovine mammary epithelial cell line, were used as the experimental model. The BME-UV1 cells were treated with complete medium containing 50 μ*M* *cis*-9,*trans*-11 CLA (c9,t11 CLA), *trans*-10,*cis*-12 CLA (t10,c12 CLA), and CLA mixture (1:1, *cis*-9,*trans*-11: *trans*-10,*cis*-12 CLA). To monitor cellular uptake of CLA isomers, cells and culture medium were collected at 0, 3, and 48 h from CLA addition for lipid extraction and fatty acid analyses. To assess the cellular antioxidant response, glutathione (GSH/GSSH), NADPH, and γ-glutamyl-cysteine ligase activity was measured after 48 h from addition of CLA. Cytoplasmic superoxide dismutase, glutathione peroxidase, glutathione S-transferase, and glutathione reductase activities and mRNA were also determined. Intracellular reactive oxygen species and thiobarbituric acid reactive substance production were assessed in cells supplemented with CLA isomers. Cell viability after 3 h to H_2O_2 exposure was assessed to evaluate and to compare the potential protection of different CLA isomers against H_2O_2-induced oxidative stress. Mammary cells readily picked up all CLA isomers, their accumulation was time dependent, and main metabolites at 48 h are two 18:3 isomers. The CLA treatment induced an intracellular GSH increase, matched by high concentration of NADPH, and an increase of γ-glutamyl-cysteine ligase activity mainly in cells treated with the t10,c12 CLA isomer. The CLA isomer treatment of bovine mammary cells increased superoxide dismutase, glutathione peroxidase, and glutathione S-transferase activity and decreased glutathione reductase activity, but no changes in gene expression of these antioxidant enzymes were observed. Cells supplemented with CLA isomers showed a reduction in intracellular reactive oxygen species and thiobarbituric acid reactive substance levels. All CLA isomers were able to enhance cell resistance against H_2O_2-induced oxidative stress. These suggest an antioxidant role of CLA, in particular of t10,c12 CLA, by developing a significantly high redox status in cells.
Key words: *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, bovine mammary gland cell, oxidative stress

INTRODUCTION

In dairy cows, the transition period is particularly important for health and subsequent performance of these animals, which are exposed to drastic physiological changes and metabolic stress (Grummer, 1993; Goff and Horst, 1997; Drackley, 1999). Usually, cows in this transition phase are secreting more energy than they consume; as a consequence, animals experience a substantial negative energy balance and often even oxidative stress (Bernabucci et al., 2005). Substantial evidence corroborates that oxidative stress during the prepartum and early lactation period may contribute to several health disorders in dairy cattle (Bernabucci et al., 2005; Sordillo et al., 2009). A management method used in dairy cows to alleviate the magnitude of negative energy balance is feeding rumen-protected CLA. The influence of CLA on milk composition has been evaluated in several studies, and it has been shown that *trans*-10,*cis*-12 CLA treatment results in milk fat depression, whereas *cis*-9,*trans*-11 CLA was not effective in milk fat depression (Baumgard et al., 2000a; Bauman et al.,

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2008). Some authors (Baumgard et al., 2000a,b; Mackle et al., 2003) reported that *trans*-10,*cis*-12 have little or no effect on basal plasma concentrations of metabolites (glucose, NEFA, BHBA) and hormones (leptin, insulin, IGF-I) associated with energy homeostasis. In contrast, other studies demonstrated a significant effect of CLA supplementation (as a mix of *trans*-10,*cis*-12 and *cis*-9,*trans*-11 CLA) on circulating IGF-1 and adiponectin in transition dairy cows, resulting in a prolongation of peripartal insulin resistance and drain of nutrients toward the mammary gland (Castañeda-Gutiérrez et al., 2005; Singh et al., 2014).

The effects of *trans*-10,*cis*-12 CLA in dairy cows appear to be specific to the mammary gland. Moreover, *trans*-10,*cis*-12 CLA effects may depend on the dietary status and the stage of lactation of the studied cows (Bauman and Griinari, 2003; Selberg et al., 2004; Castañeda-Gutiérrez et al., 2005; Odens et al., 2007). Hötger et al. (2013) reported that CLA supplementation (as a mixture of *trans*-10,*cis*-12 and *cis*-9,*trans*-11 CLA) in dairy cows reduces endogenous glucose production during early lactation, most likely due to lower glucose utilization for milk fat synthesis and a more efficient whole-body energy utilization in CLA-fed cows. Pappritz et al. (2011) as well as von Soosten et al. (2012) concluded that *trans*-10,*cis*-12 CLA-fed cows have an increased efficiency of ME utilization.

Given that the administration of *trans*-10,*cis*-12 CLA in mammary cells induces a reduction in the synthesis of milk fat, and this may leads to an increased availability of glucose, we hypothesized that glucose sparing might be used for cellular activities related to oxidative response other than lactose and milk synthesis. To date, no information is available concerning the relationship between administration of CLA isomers and changes in oxidative status of the bovine mammary gland. Some studies have shown the protective effects of CLA isomers (mainly *cis*-9,*trans*-11 CLA) against oxidative stress and lipid peroxidation in animal models (Andreoli et al., 2010; Chinnadurai et al., 2013). On the other hand, adverse effects of *trans*-10,*cis*-12 CLA regarding increased susceptibility to lipid autoxidation and enhancing FA oxidation have also been reported in 3T3-L1 cells and in animal models (Da Silva Marineli et al., 2012; den Hartigh et al., 2013). However, mechanism of action is not well understood, yet. Therefore, the study aims to assess in vitro effects of *trans*-10,*cis*-12 and *cis*-9,*trans*-11 CLA isomers on the cellular antioxidant response of bovine mammary cells, to examine whether these CLA isomers could play a role in cell protection against the oxidative stress and to study the molecular mechanism involved.

MATERIALS AND METHODS

Cell Culture Conditions

The BME-UV1 cell line was created at the University of Vermont from primary bovine mammary epithelial cells in culture by stable transfection with SV-40 large T-antigen (Zavizion et al. 1996). The BME-UV1 cells were kindly provided by Antonella Baldi, Department of Health, Animal Science and Food Safety (VESPA), University of Milan, Italy. Cells were routinely cultivated into 75-cm^2 tissue culture flasks (Costar, Corning, NY), in a mixture of 50% DMEM-F12, 30% RPMI-1640, and 20% NCTC-135 (Sigma-Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum, 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 m*M* glutathione, 1 μg/mL of insulin, 5 μg/mL of transferrin, 1 μg/mL of hydrocortisone, 0.5 μg/mL of progesterone, 10 μg/mL of L-ascorbic acid, and antibiotics (penicillin, 100 IU/mL; streptomycin, 100 μg/mL). All medium supplements were from Sigma-Aldrich. The cells were maintained at 37°C in a humidified 5% CO_2 incubator until confluence. Cells used in the present work were at passage numbers between 39 and 41.

Experimental Design

To examine the role of CLA on oxidative status of bovine mammary gland, BME-UV1 cells were resuspended in complete culture medium to a concentration of 5×10^5 cells/mL and dispensed into wells of 6-well and 96-well tissue culture plates. After 24 h of incubation at 37°C in an atmosphere of 5% CO_2, the medium was removed and cells were treated with complete medium containing 50 μ*M* *cis*-9,*trans*-11 CLA (**c9,t11 CLA**), *trans*-10,*cis*-12 CLA (**t10,c12 CLA**), and CLA mixture (50% c9,t11 and 50% t10,c12 CLA). To monitor cellular uptake of CLA isomers, cells were treated as described above and samples of cells and culture medium were collected at 0, 3, and 48 h from CLA addition for lipid extraction and FA analyses.

To study the cellular antioxidant response, the role of CLA in cell protection against the oxidative stress and the molecular mechanism involved, cell samples were collected for cell extracts and mRNA extraction after 48 h from addition of CLA. To test the potential protection of CLA against hydrogen peroxide (H_2O_2)-induced oxidative stress, cells were treated as described above followed by incubation at 37°C for 3 h with H_2O_2 (50 and 100 μ*M*) and cell viability was determined. The CLA concentration (50 μ*M*) was used because at 50 μ*M* concentration, no differences were observed in cell viability between CLA treatments and control. Moreover,

previous studies have shown that higher concentrations of CLA induce apoptosis in the mammary cells (Keating et al., 2008; Wang et al., 2014). Finally, the physiological concentration of CLA in human sera is in the range of 10 to 70 μM (De la Torre et al., 2005). Therefore, the concentration of 50 μM used in the present study is close to physiological levels. The CLA isomers were purchased from Larodan (Malmo, Sweden).

The experiments included at least 3 replicates per treatment and were repeated at least twice.

CLA Uptake by Cells

Lipid Extraction. Total lipids of cell pellet and culture medium were extracted according to a modified Folch et al. (1957) procedure. Briefly, the whole cell pellet (almost 1 million cells) or 0.1 mL of culture medium were dissolved in a screw-cap tube with 12 mL of chloroform/methanol (2/1 vol/vol) with 2 μg of vitamin E added and left in the dark at room temperature for 60 min. Then 4 mL of water was added and tubes were centrifuged at 900 × *g* for 60 min at 4°C to obtain 2 phases. After centrifugation the lower phase containing chloroform and total lipids was collected. The extracted fat was dried at 30°C by means of a rotary evaporator device.

Preparative Procedure for HPLC Analysis. Preparation of FFA was obtained by mild saponification according to Melis et al. (2001): lipid extracts were dissolved in 5 mL of ethanol, 0.1 mL of desferal in water (25 mg/mL), 1 mL of a 25% water solution of ascorbic acid, 0.5 mL of 10 *N* KOH, left in the dark at room temperature for 14 h. Then 10 mL of n-hexane and 7 mL of water were added and the solution was acidified by adding 37% HCl, to a pH 3 to 4. Samples were vortexed for 1 min, then centrifuged at 900 × *g* for 60 min at 4°C. Finally, solvent was evaporated and the residue was dissolved in 0.5 mL of a mixture acetic acid 0.14% (vol/vol) in acetonitrile.

Diode Array Detector HPLC Analysis of FFA. Analysis of FFA was carried out with a Varian Dual Prostar 210 liquid chromatograph (Varian, Palo Alto, CA) equipped with a diode array detector. A OmniSpher 5 C18 column, 5 μm particle size (Chrompack, Middelburg, the Netherlands), 250 × 4.6 mm was used with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, vol/vol/vol) and a flow rate of 1.5 mL/min. Unsaturated nonconjugated FA were detected at 200 nm, conjugated diene FA at 234 nm. The CLA isomers were recognized by using a commercial standard mixture of c9,t11 CLA and t10,c12 CLA and pure c9,t11 CLA or t10,c12 CLA standards (Matreya Inc., Pleasant Gap, PA). Because CLA metabolites are not commercially available, their recognition was obtained by diode array spectra and by comparison with published HPLC profiles (Melis et al., 2001).

Cellular Antioxidant Response and Cell Protection

Thiol Redox Status and Antioxidant Enzymes. The thiol redox status of BME-UV1, cultured under the conditions described above, was assessed by measuring the content of glutathione (**GSH/GSSH**), the content of nicotinamide adenine dinucleotide phosphate (NADPH), and the activity of γ-glutamyl-cysteine ligase (**γGCL**).

Cytoplasmic superoxide dismutase (**SOD**), glutathione peroxidase (**GPx1**), glutathione S-transferase (**GST**), and glutathione reductase (**GR**) activities were determined in BME-UV1 cultured under the conditions described above and were measured using colorimetric assays.

For the determination of GSH/GSSG and NADPH, adherent cells were detached using trypsin/EDTA solution and centrifuged at 4,500 × *g* for 5 min at 4°C. The pellet was resuspended in 200 μL of PBS and lysed by sonication at 100 W for 60 s, centrifuged (15,000 × *g* for 5 min at 4°C), and stored at −20°C until analysis. In cell extracts, NADPH and GSH/GSSH concentrations were determined by colorimetric assays (BioAssay Systems, Hayward, CA). Optical density was measured by a spectrophotometer at 405 and 540, respectively.

The γGCL activity was determined by a fluorescence assay as described by Chen et al. (2010). The BME-UV1 cells were detached using trypsin/EDTA solution and centrifuged at 4,500 × *g* for 5 min at 4°C. The pellet was resuspended in 100 μL of TES/SB buffer (wt/vol, 1/4) consisting of 20 m*M* Tris, 1 m*M* EDTA, 250 m*M* sucrose, 20 m*M* sodium borate, and 2 m*M* serine. The cells were sonicated at 100 W for 60 s and then centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatants were collected and centrifuged again at 15,000 × *g* at 4°C for 20 min. The supernatants were collected and the protein concentrations were determined using a BCA Protein Assay Kit from Pierce (Rockford, IL), with BSA used as the standard. For the γGCL activity assay, aliquots of 30 μL supernatant were mixed with 30 μL of γGCL reaction cocktail (400 m*M* Tris, 40 m*M* ATP, 40 m*M* L-glutamic acid, 2 m*M* EDTA, 20 m*M* sodium borate, 2 m*M* serine, and 40 m*M* $MgCl_2$). Following incubation at 37°C for 5 min, 30 μL of cysteine solution (30 m*M*; dissolved in TES/SB buffer) was added and the mixtures were incubated at 37°C for 13 min. The enzymatic reaction in the mixture, was stopped by precipitating proteins with 200 m*M* 5-sulfosalicylic acid. After placing on ice for 20 min, the mixtures were centrifuged at 2,000 × *g* at 4°C for 10 min. Following centrifugation, 20 μL of each superna-

tant containing γ-glutamylcysteine (**γGC**) was added to a 96 well plate designed for fluorescence detection. For each assay, 20 μL of γGC standards, containing 5 μL of γGCL reaction cocktail [5 μL of 5-sulfosalicylic acid (200 m*M*), 5 μL of H_2O, and 5 μL of γGC standard solution (0, 20, 40, 60, 80, 100, 120, and 140 μ*M* in TES/SB buffer)], was added to each well of the same 96-well plate to generate a standard curve. Next, 180 μL of 2,3-naphthalenedicarboxyaldehyde (**NDA**) was added to each well. Following incubation in the dark at room temperature for 30 min, the formation of NDA-γGC was measured (472 nm excitation/528 nm emission) using a fluorescent plate reader (Multimode Detector DTX 880, Beckman Coulter Inc., Fullerton, CA). The production of γGC in each sample was calculated using the standard curve. Values were expressed in micromoles per microgram of protein.

To determine GPx-1, GST, GR, and SOD activity, after incubation, the plates were placed in ice, the monolayers were mechanically detached with a scraper and the cells were sonicated (100 W for 60 s), centrifuged (15,000 × *g* for 5 min at 4°C), and stored at −80°C until analysis. The enzymatic activities were determined by colorimetric assays (BioAssay Systems, Hayward, CA). Optical density was measured by a spectrophotometer at 340, 405 and 450 nm, respectively.

RNA Isolation and Real-Time PCR. The mRNA abundance of bovine antioxidant enzymes (GPx-1, GSR, GST, and SOD) was assayed in BME-UV1 cultured under the conditions described above and were carried out by real-time PCR.

To isolate total RNA, BME-UV1 cells were seeded in 6-well tissue culture plate at the concentration of 5 × 10^5 cells/mL in complete medium containing 10% fetal bovine serum. Total RNA was extracted by QIAzol Lysis Reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions and stored at −80°C. The RNA was quantified using Quant-iT RNA assay Kit (Invitrogen, Carlsbad, CA) and fluorescence was measured at excitation/emission of 644/673 nm. The integrity of the RNA was checked by visualization of 18S and 28S ribosomal bands on an agarose gel. One microgram of total RNA was reverse transcribed using a Quantitect reverse transcription kit (Qiagen) in a total volume of 20 μL on a PCR Express thermal cycler (Hybaid, Ashford, UK). Quantitative SYBR Green real-time PCR was performed following the manufacturer's recommendations using LightCycler (Roche Applied Science, Indianapolis, IN). To account for possible variation related to cDNA input or the presence of PCR inhibitors, the endogenous reference gene *RPS9* was simultaneously quantified for each sample, and data were normalized accordingly. In Table 1 the specific characteristics of primers used for the real-time PCR are shown. The PCR products were subjected to a melting curve analysis on the LightCycler and subsequently 2% agarose/Tris–borate–EDTA gel electrophoresis to confirm amplification specificity and amplicon size. To allow relative quantification after PCR, standard curves were constructed from the standard reactions for each target and housekeeping genes by plotting crossing point (**Cp**) values (i.e., the cycle number at which the fluorescence signal exceeds background versus log cDNA dilution). The Cp readings for each of the unknown samples where then used to calculate the amount of either the target or housekeeping relative to the standard, using the second derivative maximum method with the LightCycler analysis software 3.5 (Roche Applied Science).

Reactive Oxygen Species and Thiobarbituric Acid Reactive Substance Production. To determine reactive oxygen species (**ROS**) concentration, cells were washed twice with PBS and incubated with 20 μ*M* 2′,7′-dichlorodihydrofluorescin diacetate probe in PBS at 37°C for 40 min. Fluorescence was measured at 485 nm (excitation) and 535 nm (emission) wavelengths on a microplate reader (Multimode Detector DTX 880, Beckman Coulter Inc.).

For the determination of thiobarbituric acid reactive substances (**TBARS**), adherent cells were detached

Table 1. DNA sequences of bovine sense and antisense primers used for real-time PCR analysis

Gene[1]	Primer	Temperature of annealing, °C	Sequence
GPx1 forward	CTTCCCCTGCAACCAGTTTG	60	NM_174076
GPx1 reverse	GGCAATTCAGGATCTCCTCGTT		
GR forward	GACCAAACCATCGAAGGAGAGTAT	60	NM_001114190
GR reverse	CTCGTCCAAGCATCTCTTCCT		
CuZn SOD forward	GAAGAGAGGCATGTTGGAGA	57	NM_174615
CuZn SOD reverse	CCAATTACACCACGAGCCAA		
GST1 forward	AATGGACGTGGCAGAATGGAGT	60	NM_177515
GST1 reverse	GGGCACAGTGGTAAATGCATGA		
RPS9 forward	GGCGGCTCGTCCGTATC	60	XN_864261
RPS9 reverse	AATCTTCAGGCCCAGGATGTAATC		

[1]GPx1 = glutathione peroxidase, GR = glutathione reductase, GST = glutathione-S-transferase, and SOD = superoxide dismutase.

using trypsin/EDTA solution and centrifuged at 4,500 × *g* for 5 min at 4°C. The pellet was resuspended in 200 μL of PBS and lysed by 2 cycles of sonication at 100 W for 30 s, centrifuged (15,000 × *g* for 5 min at 4°C), and stored at −20°C until analysis. In cell extracts TBARS concentrations were determined by colorimetric assays (BioAssay Systems, Hayward, CA). Optical density was measured by a spectrophotometer at 340 nm.

Cell Viability. The BME-UV1 cells were seeded into 96-well microplates at an optimal density (5×10^5 cells/mL) and were grown with different CLA isomers (c9,t11 CLA, t10,c12 CLA, and CLA mixture) for 48 h. Then cells were incubated at 37°C for 3 h with different concentrations (50 and 100 μM) of H_2O_2. Cell viability before and after incubation with H_2O_2 was determined using an XTT assay with a Cell Proliferation kit II {XTT: sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; Roche Applied Science} according to the manufacturer's instructions. For each treatment, after 3 h of exposure to H_2O_2, 50 μL of XTT labeling mixture was added to each well. After 4 h incubation at 37°C, absorbance was measured at 450 nm. Background absorbance was subtracted from each value. Results were expressed as optical density (OD_{450}).

Statistical Analysis

All data of the experiment are presented as least squares means and standard error of the means. The data were analyzed by ANOVA using Statistica-7 Software package (Stat Soft Inc., Tulsa, OK). The significance of the differences was assessed by the Fisher's least significant difference (LSD) test. Significance was declared at $P < 0.05$.

RESULTS

Incorporation of CLA Isomers in Cell Lipids

The cellular uptake of FA was determined at 3 and 48 h incubation in the presence of c9,t11 CLA, t10,c12 CLA, and CLA mix. The disappearance of the FA from the medium and the appearance in the cells was used as a measure of FA uptake. As shown in Table 2, the average cellular uptake of FA during the 3-h incubation was 23, 22.85, and 19.52% for c9,t11 CLA, t10,c12 CLA, and CLA mix, respectively. After 48 h FA uptake increased to 73.25, 71.88, and 78.60% for c9,t11 CLA, t10,c12 CLA, and CLA mix, respectively. This is also confirmed by a simultaneous decrease of CLA isomers from the culture medium. All FA were highly incorporated. Cells cultured with c9,t11 CLA led to the highest cellular contents of total FA. During the 3 and 48 h incubation, a progressive enrichment of C18:3 c6,c9,t11 and C18:3 c6,t10,c12 as CLA metabolites was observed within cells. No significance variation was noted concerning the differences between each FA for uptake rates.

Thiol Redox Status

Oxidized and Reduced Glutathione. The levels of nonenzymatic antioxidants like GSH are depicted in Figure 1. Quantitative determination of reduced and oxidized GSH (Figure 1A and B, respectively) showed an increase ($P < 0.01$) of reduced GSH in cells treated with t10,c12 CLA, c9,t11 CLA, and CLA mix compared with control; in particular, t10,c12 CLA showed an increase ($P < 0.01$) of reduced GSH compared with c9,t11 CLA and CLA mix. No differences in the level of oxidized GSH were noted for cells treated with CLA isomers compared with the control culture.

Quantitative Determination of NADPH. Concentrations of a redox coenzyme NADPH are reported in Figure 1C. Cells treated with t10,c12 CLA showed higher levels ($P < 0.01$) of NADPH compared with other CLA treatments and control.

γ-Glutamyl-Cysteine Ligase Activity. Thiol redox status was assessed by measuring γGCL activity (Figure 1D). After the 48 h, CLA isomers induced a strong antioxidant response by increasing ($P < 0.01$) intracellular γGCL activity compared with the control. No differences of γGCL activity were observed for different CLA treatments.

Quantification of Activity and mRNA Expression Levels of the Antioxidant Enzymes. The enzymatic activities of GPx1, GR, GST, and SOD in cell lysates are shown in Figure 2 (panels A, B, C and D, respectively). From this figure, it is evident that the activity of GPx1 showed an increase ($P < 0.01$) in cells treated with CLA compared with control. The GPx1 activity was greater ($P < 0.01$) in c9,t11 CLA cells with respect to t10,c12 CLA and CLA mix.

The GR activity decreased ($P < 0.01$) in cells treated with t10,c12 CLA and CLA mix compared with control cells. In cells treated with c9,t11 CLA, the GR activity was not different compared with control but showed a decrease ($P < 0.05$) compared with other CLA treatments (t10,c12 and mix).

The GST activity showed an increase ($P < 0.05$) in cells treated with t10,c12 CLA compared with control. The activity of SOD was increased ($P < 0.01$) by CLA compared with control.

In Figures 3A, 3B, 3C, and 3D, the mRNA expression of GPx1, GR, GST, and SOD, respectively, is reported. The levels of gene expression of GPx1, GR, GST, and SOD mRNA did not show any differences between CLA

Table 2. Concentration of fatty acids in BME-UV1 cells and culture medium after 3 and 48 h CLA isomer incubation[1]

	Cells, nmol/10^6 cells			Culture medium, nmol/mL		
Fatty acid[1]	Control[2]	3 h	48 h	Control	3 h	48 h
C18:2 c9,t11	0.36 ± 0.07	1.92 ± 0.76	9.11 ± 0.54	0.25 ± 0.0004	5.75 ± 0.59	3.65 ± 0.64
C18:2 c9,t11	ND	0.16 ± 0.06	1.24 ± 0.08	ND[3]	ND	ND
C18:3 c6,c9,t11						
C18:2 t10,c12	0.19 ± 0.03	2.44 ± 0.42	7.93 ± 0.67	0.03 ± 0.0004	8.21 ± 1.03	3.03 ± 0.19
C18:2 t10,c12	ND	0.18 ± 0.04	1.35 ± 0.09	ND	ND	ND
C18:3 c6,t10,c12						
50% C18:2 c9,t11 + 50% C:18:2 t10,c12 (CLA mix)						
C18:2 c9,t11	0.36 ± 0.07	0.89 ± 0.22	5.47 ± 0.57	0.25 ± 0.0004	8.56 ± 0.07	1.21 ± 0.03
C18:2 t10,c12	0.19 ± 0.03	0.73 ± 0.16	2.10 ± 0.16	0.03 ± 0.0004	3.40 ± 0.39	0.50 ± 0.03
C18:3 c6,c9,t11	ND	0.05 ± 0.01	0.46 ± 0.06	ND	ND	ND
C18:3 c6,t10,c12	ND	0.08 ± 0.01	0.68 ± 0.07	ND	ND	ND

[1]c = *cis*; t = *trans*.
[2]Cells and culture medium before incubation (time = 0) were used as control. Data are expressed as mean ± SEM (n = 6).
[3]ND = nondetectable.

treatments and control. No differences were observed among treatments with different CLA isomers (c9,t11, t10,c12, and mix).

Intracellular ROS and TBARS. The GSH is recognized as the main intracellular antioxidant, it maintains the redox status of the cell and is related to intracellular ROS levels. For this reason, intracellular ROS production was assessed in cells supplemented with CLA isomers. At 48 h (Figure 4A), ROS showed a 8.6, 10.6, and 19% reduction in cells treated with c9,t11 CLA ($P < 0.05$), t10,c12 CLA ($P < 0.05$), and CLA mix ($P < 0.01$), respectively, compared with the control. The intracellular ROS levels in cells treated with CLA mix showed a decrease ($P < 0.05$) compared with the other 2 CLA treatments.

Cell concentrations of TBARS are shown in Figure 4B. All CLA treatments reduced ($P < 0.01$) TBARS levels compared with untreated cells.

Protection of Cells Against Oxidative Stress

Cell viability was carried out using the XTT assay and was evaluated after 48 h of CLA exposure. As shown in Table 3, no CLA treatments reduced the cell viability, and no differences were observed between treatments with different CLA isomers (c9,t11 CLA, t10,c12 CLA, and CLA mix).

To evaluate and to compare the potential protection of different CLA isomers against H_2O_2-induced oxidative stress, we assessed cell viability by the XTT test after 3 h to H_2O_2 exposure. As shown in Table 3, all CLA isomers were able to enhance cell resistance against oxidative stress at 48 h. Cell resistance with c9,t11 CLA, t10,c12 CLA, and CLA mix increased to 122, 114, and 126%, respectively, compared with the control.

DISCUSSION

The present study investigated the effect of the main biologically active CLA isomers, c9,t11 and t10,c12 CLA, on the cellular antioxidant response of bovine mammary gland epithelial cells (BME-UV1) and examined whether CLA isomers could play a role in cell protection against oxidative stress.

The CLA supplementation increased their concentration in the cells, and BME-UV1 cultured with CLA treatments did not produce differences in uptake rates of different CLA isomers. Therefore, the uptake rate of the tested FA was not influenced by their geometric structures. Results reveal that mammary cells readily picked up the tested FA. Accumulation of total CLA metabolites in CLA treated cultures were time dependent, and the main metabolites were 18:3 isomers (C18:3

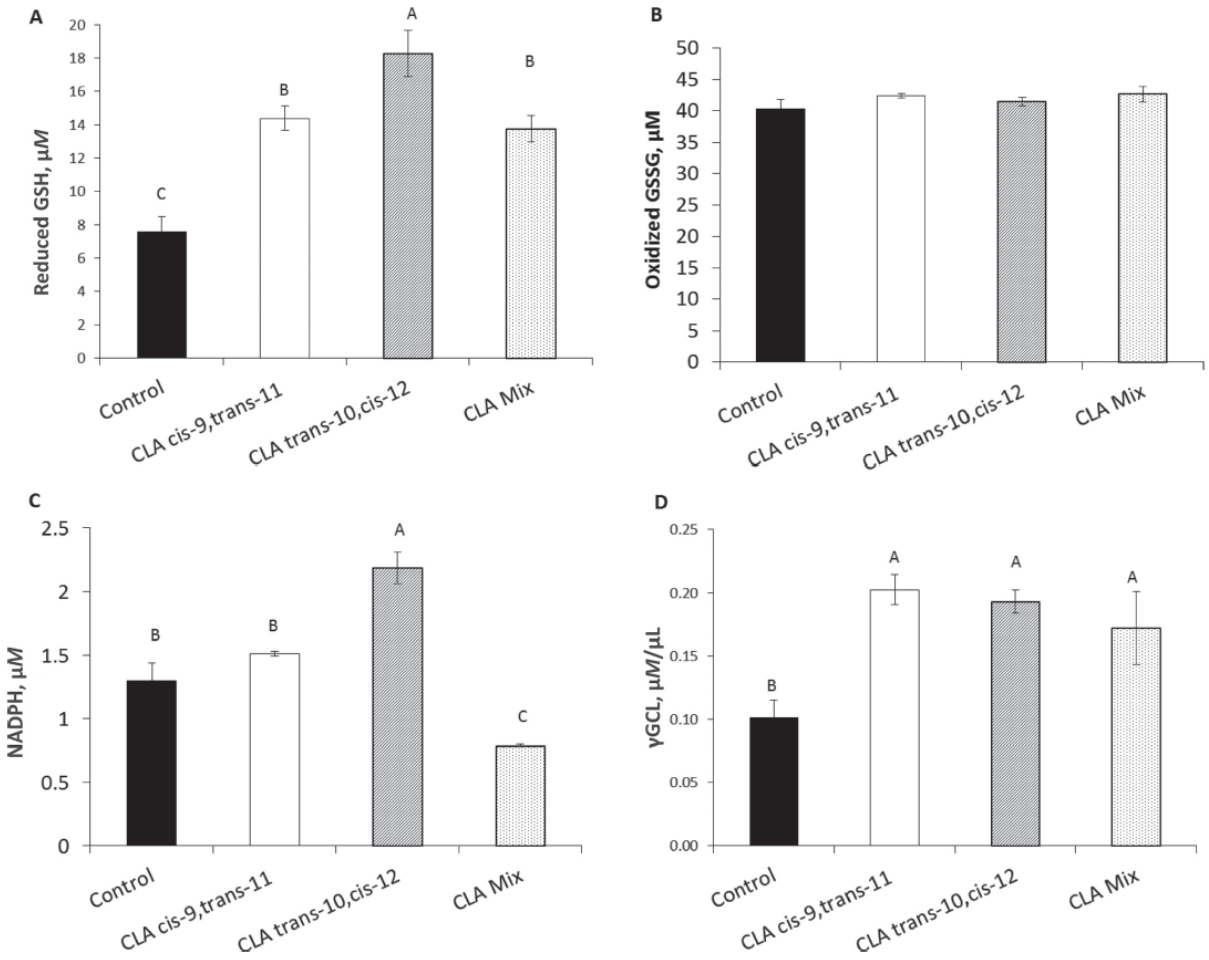

Figure 1. Intracellular concentrations of reduced (GSH; A) and oxidized (GSSG; B) glutathione, NADPH (C), and γ-glutamate cysteine ligase (γGCL; D) activity in BME-UV1 cells after 48 h of CLA isomer exposure. Data are reported as least squares means ± standard error of the mean (n = 6). Significant differences between the control group and treatments and among treatments are represented by different letters ($^{A,B}P < 0.01$). CLA mix = 50% *cis*-9,*trans*-11/50% *trans*-10,*cis*-12.

c6,c9,t11 and C18:3 c6,t10,c12). To date, this is the first time that metabolites of delta-6-desaturation of CLA isomers are identified in bovine mammary epithelial cells. These findings are in agreement with Meadus et al. (2014) who identified metabolites of t10,c12 and c9,t11 CLA after their addiction to 3T3-adipocyte cultures and observed a delta-6-desaturase (D6D∼Fads2) activity, which added a c6 double bond to the CLA isomers to make 18:3 isomers. Those authors reported that the different CLA treatments formed unique metabolites and the c9,t11 CLA isomer was preferentially converted by delta-6 desaturation (FADs2) followed by elongase (Elov5) to a conjugated 20:3-c8,c11,t13. Alternatively, the t10,c12 CLA was only desaturated to conjugated 18:3-c6,t10,c12, possibly being an unsuitable substrate for further elongation (Banni et al., 2004). In rodents and in humans, metabolites originated by peroxisomal β-oxidation of CLA isomers have also been reported (Banni et al., 2004). In the present study, conjugated 16:2 isomers were not identified, probably due to the short time of incubation.

To reduce oxidative damage, the cells modulate the redox status as a cytoprotective strategy. Results of the present study clearly showed that the exposure of cells to 50 μ*M* CLA isomers for 48 h produced a significant intracellular GSH increase, matched by high concentration of NADPH and an increase of γ-glutamylcysteine ligase activity. This was mainly observed in cells

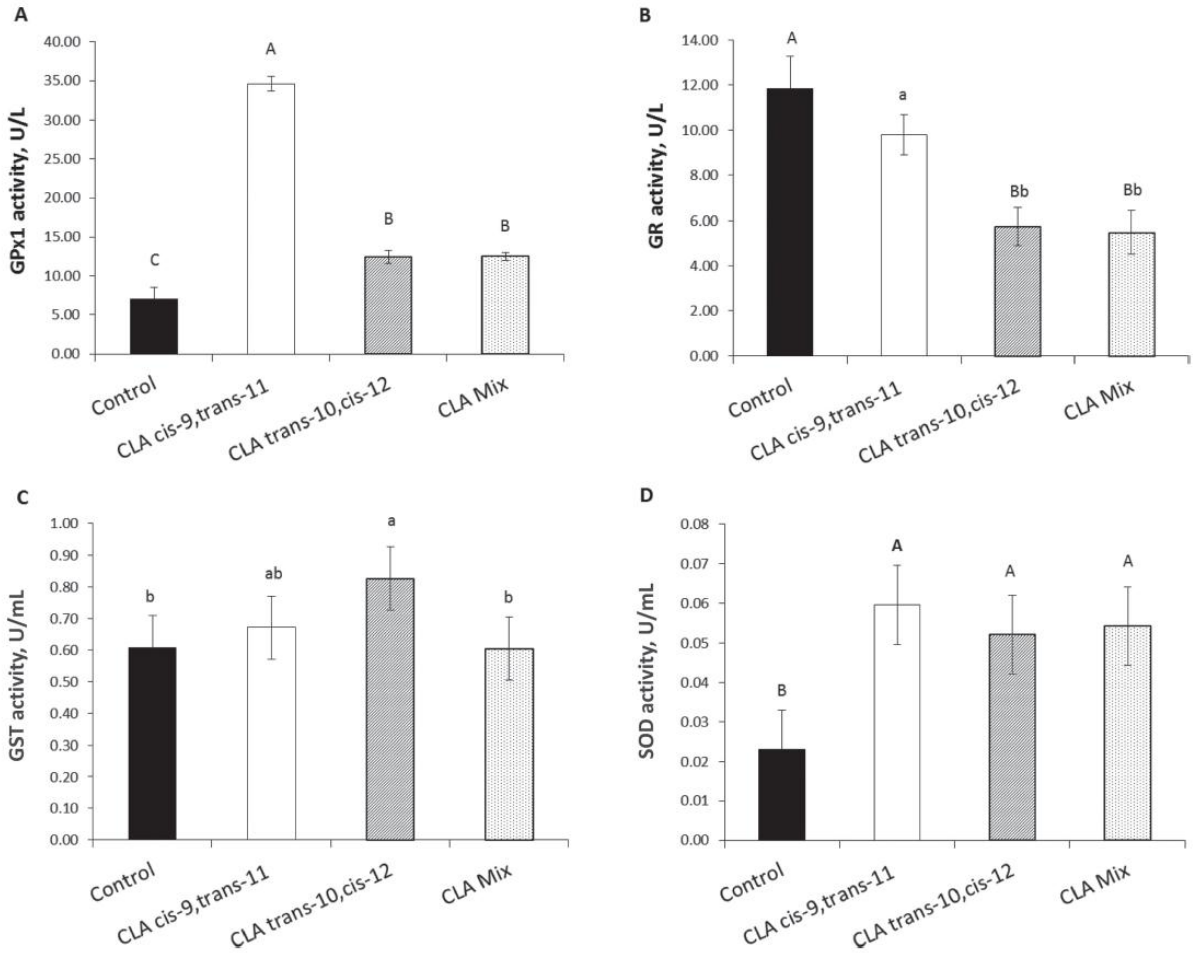

Figure 2. Intracellular levels of glutathione peroxidase (GPx1; A), glutathione reductase (GR) (B), glutathione-S-transferase (GST; C), and superoxide dismutase (SOD; D) activity in BME-UV1 cells after 48 h of CLA isomer exposure. Data are reported as least squares means ± SEM (n = 6). Significant differences between the control group and treatments and among treatments are represented by different letters ($^{A,B}P < 0.01$; $^{a,b}P < 0.05$). CLA mix = 50% *cis*-9,*trans*-11/50% *trans*-10,*cis*-12.

treated with t10,c12 CLA isomer. It was difficult to understand the low concentration of NADPH in CLA mixture treated cells.

In this study, CLA ability to elicit cytoprotective effects (enhancement of cellular GSH and NADPH availability, and γGCL activity) was shown for the first time in bovine mammary gland, and this is in agreement with literature data showing the antioxidant potential property of CLA (Jang et al., 2004; Fagali and Catalá, 2008; Chinnadurai et al., 2013). Similar results were obtained also by Arab et al. (2006) in an in vitro study using human skin fibroblasts treated with 8 different FA, including c9,t11 CLA. In that study, GSH synthesis was upregulated by c9,t11 CLA, mainly through an induction of γGCL. The GSH plays a primary role in the maintenance of intracellular redox homeostasis by affording protection against reactive oxygen and nitrogen species as well as electrophilic xenobiotics. Under normal cellular redox conditions, the major portion of this redox regulator is in the form of GSH, whereas GSSG concentration increases during oxidative stress. Replenishment of depleted GSH levels during oxidative stress, by de novo synthesis, is of great physiological relevance due to the multiplicity of the functional roles of GSH (Weijl et al., 2004). Its homeostasis is modulated to a large extent by 2 enzymes: γ-glutamylcysteine cysteine ligase, which catalyzes the committed step in GSH biosynthesis, and γ-glutamyltranspeptidase, which initi-

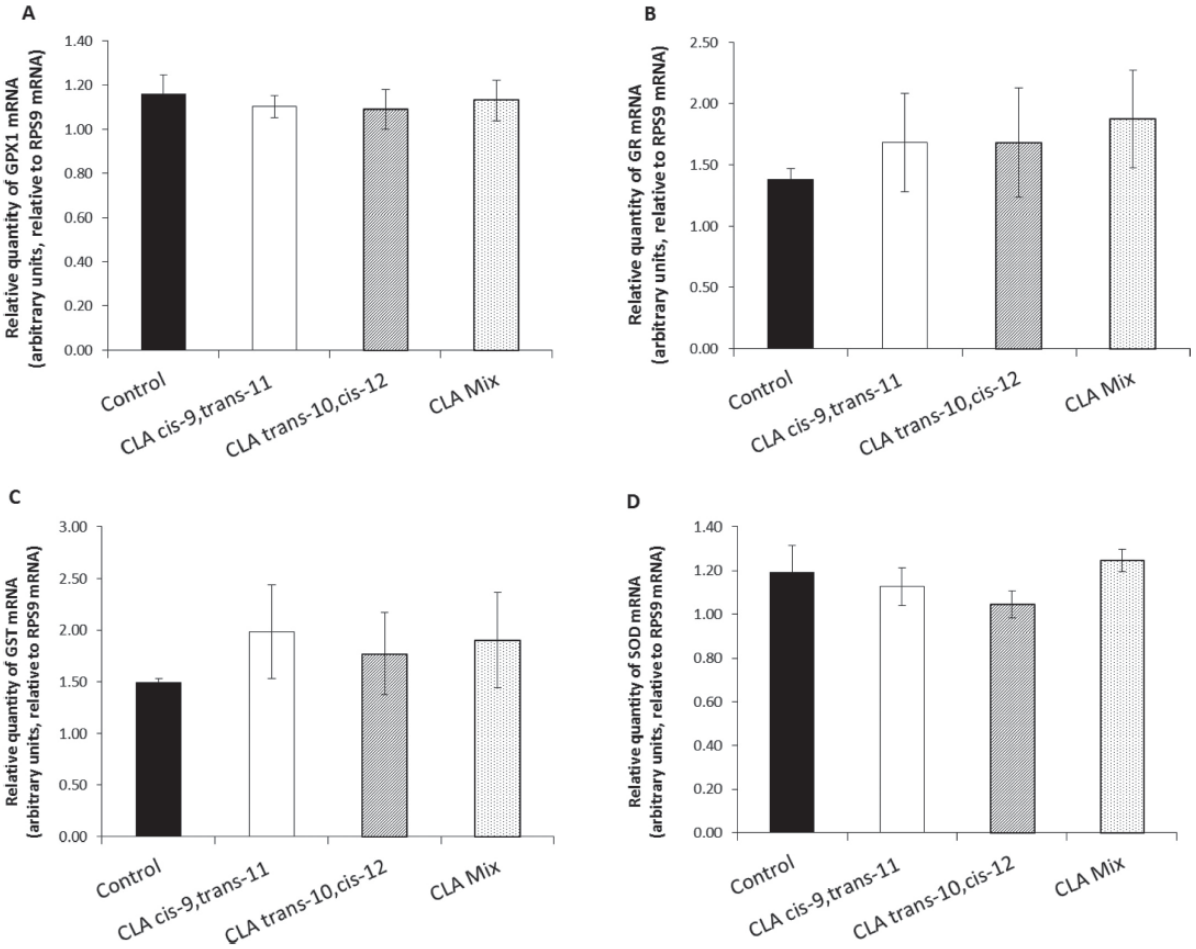

Figure 3. Messenger RNA abundance of glutathione peroxidase (GPx1; A), glutathione reductase (GR; B), glutathione-S-transferase (GST; C), and superoxide dismutase (SOD; D) in BME-UV1 cells after 48 h of CLA isomer exposure. Data are reported as least squares means ± SEM (n = 6). CLA mix = 50% *cis*-9,*trans*-11/50% *trans*-10-*cis*-12.

ates GSH reclamation. Above all an induction of γGCL is characteristic of an antioxidant response (Lee et al., 2003; Rahman, 2005). Moreover, NADPH is essential in GSH recycling and related antioxidant functions, and the ratio of the reduced to total pyridine nucleotide pool is generally accepted as an indicator of the cellular redox status (Adams et al., 2001). Toroser et al. (2006) reported that NADPH has regulatory effects on γGCL activity and increased maximal γGCL activity by up to 93% in the kidney of rats, so it may be possible to modulate de novo GSH synthesis and to control GSH homeostasis using amounts of pyridine dinucleotide phosphates.

Based on results of the present study with enhanced GSH and NADPH availability and γGCL activity, we suggest that in BME-UV1 cells the upregulated GSH synthesis by CLA may be determined by the induction of γGCL by NADPH, mainly in cells treated with t10,c12 CLA isomer. These findings are consistent with literature data indicating enhanced GSH content via the enhanced activation and induction of γGCL in human skin fibroblasts and in MRL/MpJ-Fasl mice after CLA administration (Arab et al., 2006; Bergamo et al., 2006, 2007). In particular, Arab et al. (2006) clearly demonstrated that only CLA and not other unsaturated FA were responsible in improving GSH synthesis.

In addition to nonenzymatic antioxidants as GSH, the cell can counteract oxidative stress by the antioxidant enzyme system such as SOD and various GSH-related enzymes (GPx1, GST, and GR). In the present study,

Table 3. Cell viability of BME-UV1 cells (n = 6) observed, respectively, after 48 h of CLA isomer supplementation, and after 3 h of H_2O_2 treatment (50 and 100 μM)[1]

			50 μM H_2O_2 (3 h)		100 μM H_2O_2 (3 h)	
Supplementation	LSM	SEM	LSM	SEM	LSM	SEM
Control	1.55	0.02	1.32	0.03	1.27	0.01
CLA *cis*-9,*trans*-11	1.71	0.05	1.61**	0.05	1.45**	0.04
CLA *trans*-10,*cis*-12	1.68	0.02	1.50*	0.02	1.39*	0.02
CLA mix	1.68	0.04	1.65**	0.04	1.52**	0.02

[1]Absorbance was measured at 450 nm, and data are reported as optical density (OD_{450}).
*$P < 0.05$; **$P < 0.01$: mean values were significantly different from those of the control group.

CLA isomers induced an increase of SOD, GPx1, and GST activity and a decrease of GR activity. Hence, enhancing SOD, GPx1, and GST activity the CLA treatment may offer higher protection to the cells against oxidants. Similar results were reported by in vivo studies on laboratory animals fed with CLA at high levels (Ip et al., 1991; Santos Zago et al., 2007; Chinnadurai et al., 2013). Those studies showed that administration of CLA to the diet of rats increased catalase, SOD, and GST enzyme activities, which finally led to an improvement in the antioxidative process.

The decrease of GR activity induced by CLA treatment may be ascribed to the higher presence of reduced GSH in the cells possibly due to de novo GSH synthesis by GCL, so it is not necessarily GSSG recycling to GSH.

The absence of gene expression modifications in antioxidant enzymes suggests that the CLA isomers improve the antioxidative enzyme systems through a posttranscriptional regulation. Sadi and Sadi (2010) reported that redox-sensitive proteins execute their functions via kinases, phosphatases, and transcription factors influencing the steady state levels of antioxidant enzymes. Therefore, cells may sense, transduce, and translate the oxidant signals into appropriate cellular responses depending on the cellular redox state.

Other than antioxidants, we also measured ROS and TBARS. Results indicated that the presence of CLA was associated with a low induction of ROS production. Therefore, high GSH content maintains the redox status of the cell, in this way, it is related to low intracellular ROS levels. In addition, CLA induced GSH synthesis, without any induction of ROS production. As the ability of CLA to enhance GSH content relies on γGCL induction, and the main inducers of this activity are ROS, PPAR-γ, and NADPH (Wild et al., 1999; Toroser et al., 2006). These results confirm the hypothesis that the induction of γGCL by CLA might be mediated through NADPH or, as suggested also by Arab et al.

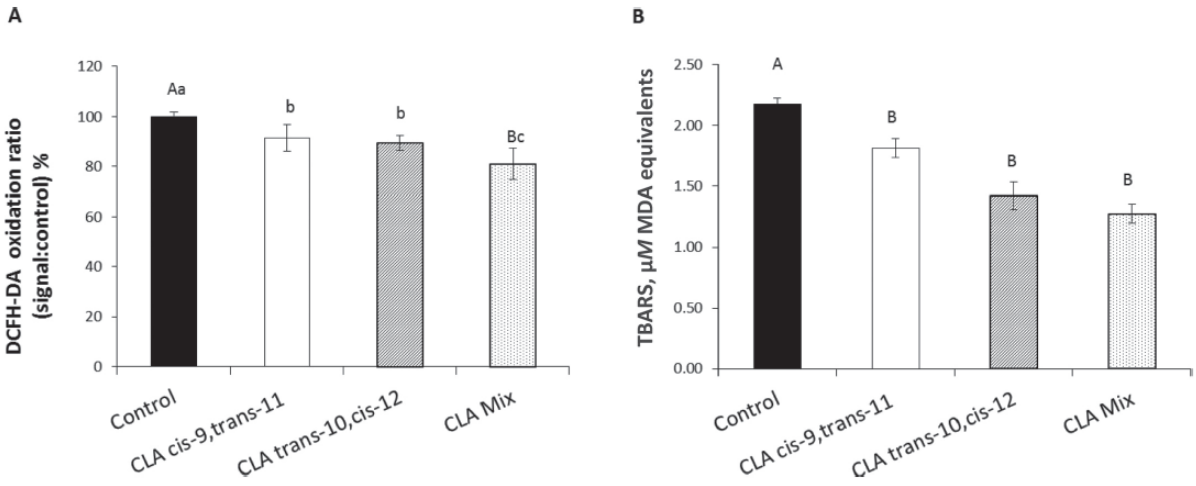

Figure 4. Intracellular production of reacting oxygen species (ROS; A) and thiobarbituric acid reactive substances (TBARS; B) level in BME-UV1 cells after 48 h of conjugated linoleic acid (CLA) isomer exposure. Data are reported as least squares means ± SEM (n = 6). Significant differences between the control group and treatments and among treatments are represented by different letters ($^{A,B}P < 0.01$; $^{a,b}P < 0.05$). CLA mix = 50% *cis*-9,*trans*-11/50% *trans*-10,*cis*-12.

(2006), through the activation of transcription factors of PPAR.

The TBARS were chosen because they have been hypothesized to represent a composite number of lipid oxidative-end products, including malondialdehyde. Therefore, the measurement of TBARS is indicative of lipid peroxidation and a biomarker of oxidative damage. In this study, CLA isomer supplementation decreased cellular concentration of TBARS. Thus, the association of this biomarker with low ROS induction seems to confirm the improvement of cell oxidative status. Fagali and Catalá (2008) clearly indicated that CLA isomers and not linoleic acid or its methyl ester provide protection against free radicals.

Finally, the measurement of cell viability in the presence of H_2O_2 was used as a further test to check cell protection. From results of the present study it is clear that CLA protected cells against oxidative damage induced by H_2O_2, and this protection seems to be mediated by the high level of GSH. In addition, Arab et al. (2006) reported that among 8 FA tested, CLA was the only PUFA able to have a protective action against oxidative insult in human skin fibroblasts.

The protective effect of a CLA-rich diet has already been described in laboratory animals (Chinnadurai et al., 2013), and the potential improving of energy balance and metabolic status observed in bovine fed with CLA-rich diets (van Knegsel et al., 2014) could be also related to the enhancement of the thiol redox status described here.

CONCLUSIONS

Findings of the present study corroborate an antioxidant role of CLA, in particular of the t10,c12 CLA isomer, by developing a significant elevated redox status in cells. In the present study, we showed that CLA isomers are potent inducers of GSH synthesis. The induction of γ-GCL by CLA could be mediated through NADPH and CLA has a protective action due to GSH synthesis without lipoperoxidation. Moreover, the enhancement of anti-oxidative capacity and cell protection against oxidative damages in mammary cells may be due to a direct effect of CLA on cell integrity. The potential role of CLA in improving energy balance and metabolic status observed in dairy cow might be also related to the enhancement of the thiol redox status described here. Moving from these findings, we propose that under physiological stress situations, as the periparturient period, the improvement of the intracellular redox homeostasis by CLA treatment could be a preparative mechanism against the relative oxidative stress caused by energy imbalance due to nutritional status, adipose mobilization, and changes in hormone profiles. Based on the data presented here, further in vivo studies are necessary to fully understand the potential role of CLA on the metabolism and antioxidant capabilities of transition cows and to show how these CLA isomers may reduce incidence of oxidative stress and related diseases in early lactation.

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REFERENCES

Adams, J. D. Jr., L. K. Klaidman, M. L. Chang, and J. Yang. 2001. Brain oxidative stress-Analytical chemistry and thermodynamics of glutathione and NADPH. Curr. Top. Med. Chem. 1:473–482.

Andreoli, M. F., P. G. Illesca, M. A. González, and C. A. Bernal. 2010. Conjugated linoleic acid reduces hepatic steatosis and restores liver triacylglycerol secretion and the fatty acid profile during protein repletion in rats. Lipids 45:1035–1045.

Arab, K., A. Rossary, L. Soulère, and J. P. Steghens. 2006. Conjugated linoleic acid, unlike other unsaturated fatty acids, strongly induces glutathione synthesis without any lipoperoxidation. Br. J. Nutr. 96:811–819.

Banni, S., A. Petroni, M. Blasevich, G. Carta, L. Cordeddu, E. Murru, M. P. Melis, A. Mahon, and M. A. Belury. 2004. Conjugated linoleic acids (CLA) as precursors of a distinct family of PUFA. Lipids 39:1143–1146.

Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Annu. Rev. Nutr. 23:203–227.

Bauman, D. E., J. W. I. Perfield, K. J. Harvatine, and L. H. Baumgard. 2008. Regulation of fat synthesis by conjugated linoleic acid: Lactation and the ruminant model. J. Nutr. 138:403–409.

Baumgard, L. H., B. A. Corl, and D. E. Bauman. 2000a. Effect of CLA isomers on fat synthesis during growth and lactation. Pages 180–190 in 62nd Proc. Cornell Nutr. Conf. Feed Manuf., Ithaca, NY.

Baumgard, L. H., B. A. Corl, D. A. Dwyer, A. Saebø, and D. E. Bauman. 2000b. Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis. Am. J. Physiol. Regul. Integr. Comp. Physiol. 278:R179–R184.

Bergamo, P., D. Luongo, F. Maurano, G. Mazzarella, R. Stefanile, and M. Rossi. 2006. Conjugated linoleic acid enhances glutathione synthesis and attenuates pathological signs in MRL/MpJ-Fas (lpr) mice. J. Lipid Res. 47:2382–2391.

Bergamo, P., F. Maurano, and M. Rossi. 2007. Phase 2 enzyme induction by conjugated linoleic acid improves lupus-associated oxidative stress. Free Radic. Biol. Med. 43:71–79.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J. Dairy Sci. 88:2017–2026.

Castañeda-Gutiérrez, E., T. R. Overton, W. R. Butler, and D. E. Bauman. 2005. Dietary supplements of two doses of calcium salts of conjugated linoleic acid during the transition period and early lactation. J. Dairy Sci. 88:1078–1089.

Chen, C. N., H. M. Brown-Borg, S. G. Rakoczy, D. A. Ferrington, and L. V. Thomposon. 2010. Aging impairs the expression of the catalytic subunit of glutamate-cysteine ligase in soleus muscle under stress. J. Gerontol. A Biol. Sci. Med. Sci. 65:129–137.

Chinnadurai, K., H. K. Kanwal, A. K. Tyagi, C. Stanton, and P. Ross. 2013. High conjugated linoleic acid enriched ghee (clarified butter) increases the antioxidant and antiatherogenic potency in female Wistar rats. Lipids Health Dis. 12:121.

Da Silva Marineli, R., A. y Castro Marques, C. P. Busch Furlan, and M. R. Maróstica Jr.. 2012. Antioxidant effects of the combina-

tion of conjugated linoleic acid and phytosterol supplementation in Sprague–Dawley rats. Food Res. Int. 49:487–493.
De la Torre, A., E. Debiton, D. Durand, J. M. Chardigny, O. Berdeaux, O. Loreau, C. Barthomeuf, D. Bauchart, and D. Gruffat. 2005. Conjugated linoleic acid isomers and their conjugated derivatives inhibit growth of human cancer cell lines. Anticancer Res. 25:3943–3949.
den Hartigh, L. J., C. Y. Han, S. Wang, M. Omer, and A. Chait. 2013. 10E,12Z-conjugated linoleic acid impairs adipocyte triglyceride storage by enhancing fatty acid oxidation, lipolysis, and mitochondrial reactive oxygen species. J. Lipid Res. 54:2964–2978.
Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: The final frontier? J. Dairy Sci. 82:2259–2273.
Fagali, N., and A. Catalá. 2008. Antioxidant activity of conjugated linoleic acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH techniques. Biophys. Chem. 137:56–62.
Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497–509.
Goff, J. P., and R. L. Horst. 1997. Physiological changes at parturition and their relationship to metabolic disorders. J. Dairy Sci. 80:1260–1268.
Grummer, R. R. 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. J. Dairy Sci. 76:3882–3896.
Hötger, K., H. M. Hammon, C. Weber, S. Görs, A. Tröscher, R. M. Bruckmaier, and C. C. Metges. 2013. Supplementation of conjugated linoleic acid in dairy cows reduces endogenous glucose production during early lactation. J. Dairy Sci. 96:2258–2270.
Ip, C., S. F. Chin, J. A. Scimeca, and M. W. Pariza. 1991. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Res. 51:6118–6124.
Jang, I. S., H. Y. Yang, and Y. H. Ko. 2004. Effect of conjugated linoleic acid on intestinal and hepatic antioxidant enzyme activity and lipid peroxidation in broiler chickens. Asian-australas. J. Anim. Sci. 17:1162–1167.
Keating, A. F., F. Q. Zhao, K. A. Finucane, D. R. Glimm, and J. J. Kennelly. 2008. Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression. Domest. Anim. Endocrinol. 34:284–292.
Lee, J. M., M. J. Calkins, K. Chan, Y. W. Kan, and J. A. Johnson. 2003. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. J. Biol. Chem. 278:12029–12038.
Mackle, T. R., J. K. Kay, M. J. Auldist, A. K. H. McGibbon, B. A. Philpott, L. H. Baumgard, and D. E. Bauman. 2003. Effects of abomasal infusion of conjugated linoleic acid on milk fat concentration and yield form pasture-fed dairy cows. J. Dairy Sci. 86:644–652.
Meadus, W. J., P. Vahmani, P. Duff, D. C. Rolland, T. D. Turner, and M. E. Dugan. 2014. Metabolism of CLA isomers, c9,t11 and t10,c12, in adipocyte cultures and their effect on delta-6 desaturase expression. Abstract no. 481 in Proc. 10th World Congress on Genetics Applied to Livestock Production. Vancouver (BC), Canada.
Melis, M. P., E. Angioni, G. Carta, E. Murru, P. Scanu, S. Spada, and S. Banni. 2001. Characterization of conjugated linoleic acid and its metabolites by RP-HPLC with diode array detector. Eur. J. Lipid Sci. Technol. 103:617–621.
Odens, L. J., R. Burgos, M. Innocenti, M. J. VanBaale, and L. H. Baumgard. 2007. Effects of varying doses of supplemental conjugated linoleic acid on production and energetic variables during the transition period. J. Dairy Sci. 90:293–305.
Pappritz, J., U. Meyer, R. Kramer, E. Weber, G. Jahreis, J. Rehage, G. Flachowsky, and S. Dänicke. 2011. Effects of long-term supplementation of dairy cow diets with rumen-protected conjugated linoleic acids (CLA) on performance, metabolic parameters and fatty acid profile in milk fat. Arch. Anim. Nutr. 65:89–107.
Rahman, I. 2005. Regulation of glutathione in inflammation and chronic lung diseases. Mutat. Res. 579:58–80.
Sadi, G., and Ö. Sadi. 2010. Antioxidants and regulation of antioxidant enzymes by cellular redox status. Turkish J. Scientific Rev. 3:95–107.
Santos-Zago, L. F., A. P. Botelho, and A. C. de Oliveira. 2007. Supplementation with commercial mixtures of conjugated linoleic acid in association with vitamin E and the process of lipid autoxidation in rats. Lipids 42:845–854.
Selberg, K. T., A. C. Lowe, C. R. Staples, N. D. Luchini, and L. Badinga. 2004. Production and metabolic responses of periparturient Holstein cows to dietary conjugated linoleic acid and trans-octadecenoic acids. J. Dairy Sci. 87:158–168.
Singh, S. P., S. Häussler, J. F. L. Heinz, B. Saremi, B. Mielenz, J. Rehage, S. Dänicke, M. Mielenz, and H. Sauerwein. 2014. Supplementation with conjugated linoleic acids extends the adiponectin deficit during early lactation in dairy cows. Gen. Comp. Endocrinol. 198:13–21.
Sordillo, L. M., G. A. Contreras, and S. L. Aitken. 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Anim. Health Res. Rev. 10:53–63.
Toroser, D., C. S. Yarian, W. C. Orr, and R. S. Sohal. 2006. Mechanisms of gamma-glutamylcysteine ligase regulation. Biochim. Biophys. Acta 1760:233–244.
van Knegsel, A. T. M., G. J. Remmelink, S. Jorjong, V. Fievez, and B. Kemp. 2014. Effect of dry period length and dietary energy source on energy balance, milk yield, and milk composition of dairy cows. J. Dairy Sci. 97:1499–1512.
von Soosten, D., U. Meyer, M. Piechotta, G. Flachowsky, and S. Dänicke. 2012. Effect of conjugated linoleic acid supplementation on body composition, body fat mobilization, protein accretion, and energy utilization in early lactation dairy cows. J. Dairy Sci. 95:1222–1239.
Wang, H., H. Liu, J. Liu, K. Zhao, C. Wang, and W. Yang. 2014. High-level exogenous *trans*10, *cis*12 conjugated linoleic acid plays an anti-lipogenesis role in bovine mammary epithelial cells. Anim. Sci. J. 85:744–750.
Weijl, N. I., T. J. Elsendoorn, E. G. Lentjes, G. D. Hopman, A. Wipkink-Bakker, A. H. Zwinderman, F. J. Cleton, and S. Osanto. 2004. Supplementation with antioxidant micronutrients and chemotherapy-induced toxicity in cancer patients treated with cisplatin-based chemotherapy: A randomised, doubleblind, placebo-controlled study. Eur. J. Cancer 40:1713–1723.
Wild, A. C., H. R. Moinova, and R. T. Mulcahy. 1999. Regulation of gammaglutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. J. Biol. Chem. 274:33627–33636.
Zavizion, B., M. van Duffelen, W. Schaeffer, and I. Politis. 1996. Establishment and characterization of a bovine mammary epithelial cell line with unique properties. In Vitro Cell. Dev. Biol. Anim. 32:138–148.

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Effect of summer season on milk protein fractions in Holstein cows

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ABSTRACT

Milk characteristics are affected by heat stress, but very little information is available on changes of milk protein fractions and their relationship with cheesemaking properties of milk. The main objective of the study was to evaluate the effect of hot season on milk protein fractions and cheesemaking properties of milk for Grana Padano cheese production. The study was carried out in a dairy farm with a cheese factory for transforming the milk to Grana Padano cheese. The study was carried out from June 2012 to May 2013. Temperature and relative humidity of the inside barn were recorded daily during the study period using 8 electronic data loggers programmed to record every 30 min. Constant managerial conditions were maintained during the experimental periods. During the experimental period, feed and diet characteristics, milk yield, and milk characteristics were recorded in summer (from June 29 to July 27, 2012), winter (from January 25 to March 8, 2013), and spring (from May 17 to May 31, 2013). Milk yield was recorded and individual milk samples were taken from 25 cows selected in each season during the p.m. milking. Content of fat, proteins, caseins (CN), lactose and somatic cell count (SCC), titratable acidity, and milk rennet coagulation properties were determined on fresh samples. Milk protein fraction concentrations were determined by the sodium dodecyl sulfate-PAGE. Data were tested for nonnormality by the Shapiro-Wilk test. In case of nonnormality, parameters were normalized by log or exponential transformation. The data were analyzed with repeated measures ANOVA using a mixed model procedure. For all the main milk components (fat, protein, total solids, and solids-not-fat), the lowest values were observed in the summer and the greatest values were observed in the winter. Casein fractions, with the exception of γ-CN, showed the lowest values in the summer and the greatest values in the winter. The content of IgG and serum albumin was greater in summer than in the winter and spring. A mild effect of season was observed for milk SCC, with greater values in summer than in the winter and spring. A worsening of milk coagulation properties was observed in summer season. The alteration of cheesemaking properties during hot season seems strictly linked with changes of milk protein fractions mainly with the decrease of α_S-CN and β-CN and the increase of undefined proteins.
Key words: heat stress, dairy cow, milk protein, casein fraction, whey protein

INTRODUCTION

Climate change is likely to be one of the main challenges that humankind faces during the current century. The increasing concern with the thermal comfort of agricultural animals is justifiable not only for countries occupying tropical zones, but also for nations in temperate zones where high ambient temperatures are becoming an issue (Nardone et al., 2010). Earth's climate has warmed in the last century (IPCC, 2007) and is predicted to continually change at rates unprecedented in recent human history [IPCC, 2007, Fourth Assessment Report (AR4)]. In our recent study (Segnalini et al., 2013), we demonstrated a gradual increase of both annual and seasonal temperature-humidity index (**THI**) during the period under investigation and a strong heterogeneity of the Mediterranean area. In particular, the analysis indicated that Spain, southern France, and Italy should be expected to undergo the highest THI increase, which in the last decade under study (2041–2050) will range between 3 and 4 units. During the summer months, the area presents characteristics indicating risk of thermal (heat) stress for farm animals.

We have to expect that the livestock systems (based on grazing, the mixed farming systems, or industrialized system) will be more and more negatively affected by climate changes (namely global warming). In addition, genetic improvement programs, which enhance production traits, may increase an animal's susceptibility to high environmental temperatures (Bernabucci et al., 2014) due to the close relationship between metabolic heat generation and production level (Kadzere et al., 2002).

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In addition to thermal effects on production, the quality of animal products is strongly and negatively affected by a hot environment. This is more important for high-quality products such as protected designation of origin (PDO) cheeses.

Milk characteristics are strongly affected by heat stress (Bernabucci et al., 2010). During hot season, a worsening of the main cheesemaking properties is observed (Calamari and Mariani, 1998); in particular, the reduction of the titratable acidity and the worsening of milk coagulation properties (**MCP**) make milk less suitable for the production of hard cheese with a long ripening time (Calamari and Mariani, 1998; Malacarne et al., 2005). Among the milk characteristics affecting the MCP, CN content and the CN composition are 2 important factors (Bertoni et al., 2001, 2005). Little information is available concerning changes of milk protein fractions (Bernabucci et al., 2002) and the relationship with cheesemaking properties of milk under heat stress conditions. Therefore, the study was aimed at evaluating the effect of hot season on milk protein fractions and cheesemaking properties of milk for Grana Padano cheese production.

MATERIALS AND METHODS

Animals and Management

The research protocol and the animal care were in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, on the protection of animals used for scientific purposes.

The study was carried-out in a dairy farm of 600 Italian Holstein lactating cows located near Brescia, Italy (45°54′N, 9°93′E; altitude 148 m above sea level). The milk yielded in this herd is used to produce Grana Padano cheese. A cheese factory is present at the farm to transform the milk in this PDO cheese. The lactating cows were raised in a free stall barn equipped with axial flow fans and sprinklers in the feeding area and in the holding pen. Sprinklers were placed perpendicular to the airflow of the fans along the feed alley. Fans and sprinklers were thermostatically controlled: fans were switched on at 23°C and sprinklers at 27°C (soaking for 1 min and ventilation for 5 min).

Cows were milked twice daily (0300 and 1500 h) and milk yield (**MY**) at each milking was recorded electronically using Afimilk meters (S.A.E. Afikim, Israel). Cows were fed using TMR technique once daily ad libitum at 0830 h. To ensure cows had ad libitum access to the TMR, the amount offered was assessed on a daily basis with the aim of producing from 3 to 8% refusal.

Constant managerial conditions (operators, similar batches of feed, milking frequency, and working routine) were maintained thorough the experimental period.

The study was carried out from June 2012 to May 2013. Measurements on microclimatic conditions, feed and diets, MY, and milk characteristics were carried out in summer (from June 29 to July 27, 2012), winter (from January 25 to March 8, 2013), and spring (from May 17 to May 31, 2013). The individual milk samples were taken from 25 cows selected in each season (5 measurements in summer, 2 in winter, and 2 in spring). The selected cows were balanced for parity, DIM, and MY (Table 1). The selected cows were hosted in 2 pens in each season.

Measurements and Analyses

Microclimatic Conditions. Temperature and relative humidity of the inside barn were recorded daily during the study period using 8 electronic probes connected to a data logger (Mini Data Logger 174-H, Testo, Milano, Italy) programmed to record every 30 min. Mean daily temperature and humidity and daily minimum and maximum temperature and humidity were calculated from temperature and relative humidity data recorded throughout the study. Data were used to compute a composite climatic welfare index, the THI, according to the formula of Kelly and Bond, as reported by Ingraham et al. (1979). The formula was

$$\text{THI} = (1.8 \cdot \text{AT} + 32) - (0.55 - 0.55 \cdot \text{RH}) \cdot [(1.8 \cdot \text{AT} + 32) - 58],$$

where AT was the ambient temperature expressed as degrees Celsius, and RH was the relative humidity expressed as fraction of the unit. Mean daily THI, daily minimum THI, and daily maximum THI were also calculated throughout the study.

Table 1. Number of cows and overall mean (±SD) of some characteristics of the groups involved in the study

Item	Winter	Spring	Summer
No. of animals	25	25	25
Parity	2.5 ± 0.6	2.5 ± 0.5	2.5 ± 0.4
DIM	124 ± 23	129 ± 22	125 ± 27
Milk yield, L·animal^{-1} per d	39.7 ± 6.5	39.8 ± 10.2	39.2 ± 6.8

Feeds and Diets. Representative samples of TMR were collected at each sampling day around 0930 h. Chemical composition (moisture, CP, crude fiber, NDF, starch, and ash) was determined using standard procedures. The chemical and nutritive characteristics of the diets were calculated at each sampling day.

Milk. At each sampling point, during the p.m. milking, MY was measured and representative individual samples were collected (MM85 with milk sampler, S.A.E. Afikim) from each cow. Fat, protein, CN, lactose contents, and titratable acidity were determined on fresh samples by the Fourier Transform Infrared Spectroscopy (FT 120, Foss Electric, Hillerød, Denmark). Somatic cell count was determined by an optofluorometric method on fresh samples (Fossomatic 180, Foss Electric). Furthermore, milk rennet coagulation properties were assessed as rennet coagulation time (**RCT**), curd firming rate ($\mathbf{k_{20}}$), and curd firmness ($\mathbf{a_{30}}$), using Formagraph (Foss Electric) on fresh samples. Briefly, 10 mL of milk was heated to 35°C, and 200 μL of rennet (Hansen standard 160 with 80% chymosin and 20% pepsin, Pacovis Amrein AG, Bern, Switzerland) diluted to 1.6% (wt/wt) in distilled water was added to milk at the start of the analysis to obtain 0.051 IMCU (international milk clotting units·mL^{-1}). Milk samples with RCT that did not coagulate after 31 min were classified as bad reactivity, and milk samples with RCT longer than 27 min were classified with scarce reactivity. Milk samples with RCT between 10.3 and 19 min were classified as samples having good coagulation properties.

Milk protein fraction concentrations were determined by SDS-PAGE performed according to Giacinti et al. (2013) on 12.5% slab gel (Figure 1). The fractions of milk proteins were diluted by adding an equal volume of double-concentrated sample buffer (125 m*M* Tris-HCl, pH 6.8, 4% SDS, 0.8 *M* dithiothreitol, 0.4% bromophenol blue, and 20% glycerol) and heated at 100°C for 3 min, to thermally denature proteins and to promote interactions of the proteins with SDS. Low molecular weight standards (Bio-Rad Laboratories, Watford, UK) were prepared in sample buffer. The electrophoresis was performed in a Mini-Protean III dual slab cell (Bio-Rad). Electrophoresis was performed at room temperature (≈22°C) using a voltage stepped procedure: voltage was kept constant (30 V) until the samples completely left the stacking gel and then the voltage was increased 15 V per min for 4 times. Voltage was maintained constant (90–100 V) until the tracking dye reached the bottom of the gel. Immediately after ending electrophoresis, gels were removed from the plates and placed in a staining solution containing 40% methanol, 10% acetic acid, and 0.1% Coomassie Brilliant Blue R-250. Gels were left for 30 min in the staining solution and then destained in the methanol/acetic acid solution (5% methanol, 7% acetic acid).

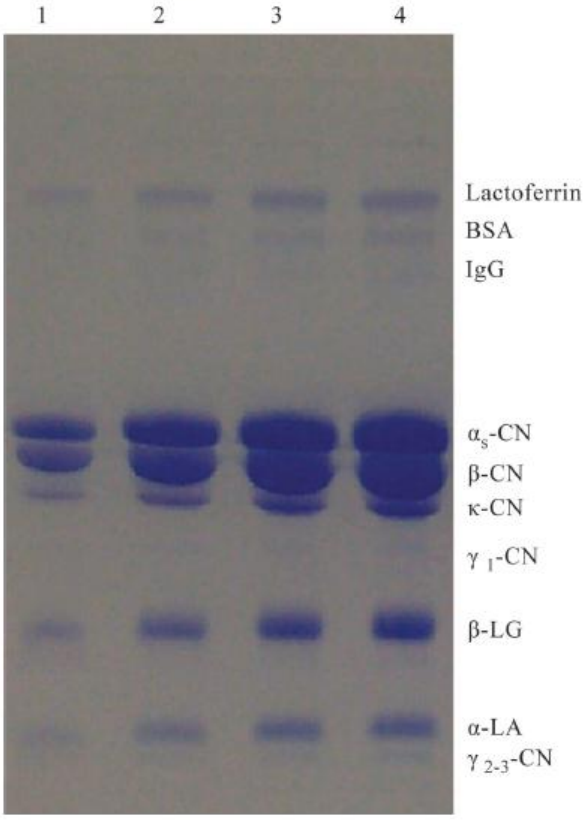

Figure 1. Representative SDS-PAGE profile of milk separated on 12.5% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Lanes 1 to 4: milk samples at different dilutions. Color version available online.

Quantitative analysis of electrophoretically separated proteins was done by densitometry using BSA (Sigma, Milano, Italy) as internal standard, with Kodak EDAS-290 densitometer and analyzed with ID Image Analysis software (Kodak Company, Rochester, NY). Detected polypeptides were identified using the standards BSA, lactoferrin, α-LA, β-LG, κ-CN, α-CN (α_S-CN), and β-CN (Sigma, Milan, Italy). All protein fractions were expressed as grams·liter^{-1}; furthermore, CN fractions were also expressed as percentage of total CN.

Statistical Analysis

All statistical analyses were performed using the statistical software package SAS 9.2 (SAS Inst. Inc., Cary, NC). Data were tested for nonnormality by the

Shapiro-Wilk test. In case of nonnormality, parameters were normalized by log or exponential transformation. The data were analyzed with repeated measures ANOVA using a mixed model (MIXED procedure of SAS). The model included the fixed effect of season (winter, spring, and summer), pen (2 levels), and the effect of sampling day, and individual variability (within cow × season). The analysis was carried out using 3 covariance structures: autoregressive order, compound symmetry, and spatial power structure. These were ranked according to their Akaike and Schwarz Bayesian information criterion, with the one having the least information criterion being eventually chosen. For each treatment, least squares means were computed, and pairwise comparisons (PDIFF option of SAS) were conducted when the F-test of one of the season factors was significant at $P < 0.10$. Statistical significance was designated as $P < 0.05$, and tendencies were declared at $P < 0.10$. The statistical analysis of protein fractions was also performed including the logarithm of SCC as a covariate in the statistical model described before.

Pearson correlations among milk variables were calculated using the CORR procedure of SAS. Furthermore, milk variables were processed by principal components (**PC**) analysis of SAS.

RESULTS

The changes of THI observed during the trial are shown in Figure 2. Summer season was characterized by elevated daily THI. In our previously study, carried out in Italian dairy cows, thresholds indicating the beginning of heat stress were found at THI values of 75, 72, and 72 for milk, protein, and fat yield, respectively (Bernabucci et al., 2014). Mean daily THI was lower to 75 THI during about 50% of summer days investigated, whereas THI was lower to the value of 72, which is the threshold indicated for fat and protein yield, for only 2 to 3 d during summer (Figure 2C). Furthermore, during summer, 3 heat waves (a period of at least 3 consecutive days during which recovery hours, with THI values below 72, were less than 10; Hahn and Mader, 1997) were detected. In spring, the average mean daily THI was 64.7 ± 3.9 (mean and SD) and the average maximum daily THI was 69.4 ± 3.8.

The average values of chemical and nutritive characteristics of the diets for each season are shown in Table 2. The chemical and nutritive characteristics of the diet were almost constant throughout the seasons. Only CP has shown a relative variability, with grater values in the winter and spring seasons.

Average MY was (means and SD) 35.7 ± 8.7, 39.7 ± 8.0, and 37.9 ± 8.0 L·animal^{-1} per d in winter, spring, and summer, respectively. For all the main milk

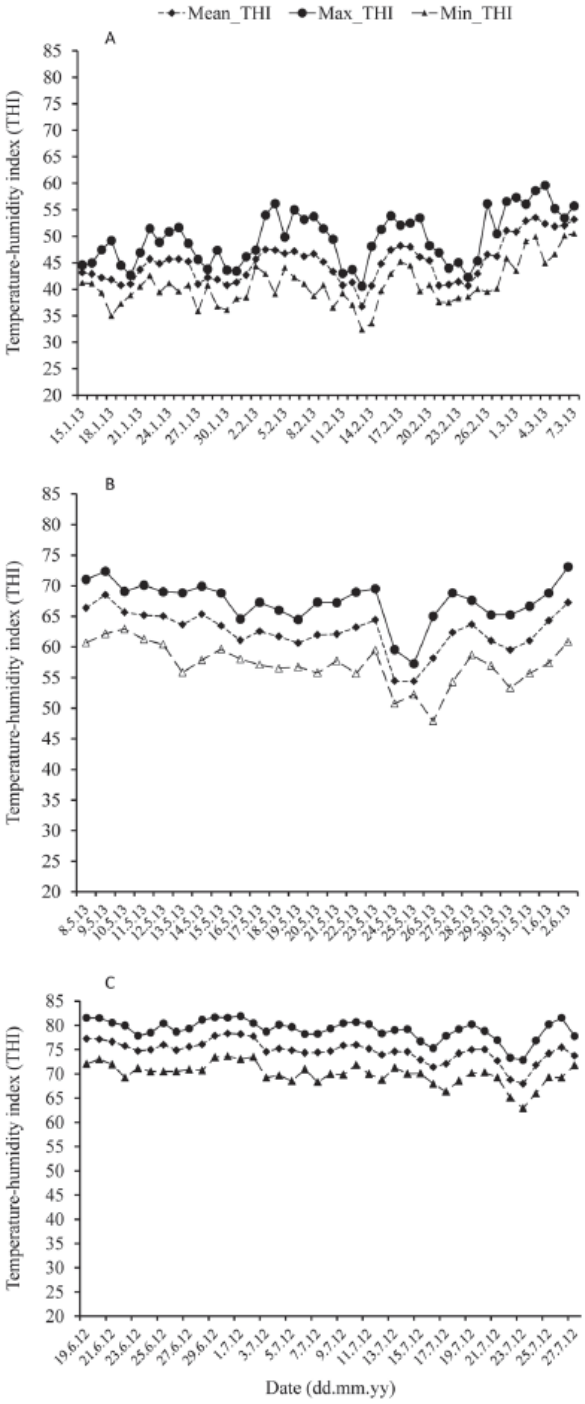

Figure 2. Microclimate conditions of barn during the experimental period: (A) winter, (B) spring, and (C) summer.

Table 2. Chemical analysis and nutritive value of diets fed to cows during winter, spring, and summer season (mean ± SD)[1]

Item	Winter	Spring	Summer
DM, %	51.48 ± 2.00	52.66 ± 0.65	52.42 ± 1.88
CP, % of DM	18.11 ± 0.97*	17.50 ± 1.11	15.74 ± 0.66
Ash, % of DM	6.57 ± 0.26	5.64 ± 0.16	5.96 ± 0.61
Crude fiber, % of DM	18.36 ± 1.55	16.88 ± 1.03	17.10 ± 1.30
NDF, % of DM	31.70 ± 0.51	31.25 ± 0.91	32.29 ± 1.61
Starch, % of DM	28.14 ± 1.09	31.49 ± 0.46*	28.27 ± 0.73

[1]On average, the mineral and vitamin supplementation was (per animal per day): 93.3 g of Ca, 16.0 g of P, 20 g of Mg, 62 g of Na, 81 mg of I (KI); 1,027 mg of Mn (MnO); 100 mg of Cu ($CuSO_4 \cdot 5H_2O$); 2,013 mg of Zn ($ZnSO_4 \cdot H_2O$ and ZnO); 4.5 mg of Se (Na_2SeO_3); 500,000 IU of vitamin A; 55,000 IU of vitamin D_3; and 1,100 mg of vitamin E acetate (α-tocopheryl acetate).

*Asterisks indicate significant differences from the value observed in the summer season ($P < 0.05$).

components (total solids, SNF, fat, and proteins), the lowest values were observed in summer season and the greatest in winter; intermediate values were observed in spring. Among the main milk components, only lactose did not change throughout the seasons (Table 3). Total solids in summer were 5.3% lower ($P = 0.010$) than in the winter and were 3.5% lower ($P = 0.020$) than in the spring. Similarly, fat percentage in summer was 15.8% lower ($P = 0.001$) than in the winter and was 11.1% lower ($P = 0.011$) than in the spring. Solids-not-fat percentage was 2.8% lower during summer ($P = 0.012$) than in the winter, and was only numerically lower (1.7% lower) than in the spring. Similarly protein percentage in summer was 6% lower ($P = 0.070$) than in the winter, and was 3.2% lower ($P = 0.049$) than in the spring.

A mild effect of season ($P = 0.070$) was observed for milk SCC (Table 3), with greater values in summer (151,000 cells·mL^{-1}) than in the winter (72,000 cells·mL^{-1}) and in the spring (69,000 cells·mL^{-1}). The percentage of milk samples with SCC greater than 400,000 cells·mL^{-1} was 25% in the summer and 10% in the winter and spring, respectively.

A worsening of MCP was observed in summer season (Table 3). In this season, the milk was characterized by a longer RCT to begin coagulation after the addition of the clotting enzyme. Also the k_{20} worsened in summer season compared with winter and spring, with a numerically longer time to reach a curd firmness of 20 mm in summer season. Furthermore, a_{30} tended to be lower in summer compared with winter and spring ($P = 0.071$ and $P = 0.079$, respectively). The percentage of milk samples with scarce or very bad reactivity (milk with RCT longer than 27 min or milk that did not coagulate after 31 min) was greater in summer (25%) than in the winter (12%) and spring (15%). The percentage of milk samples having good coagulation properties was lower in summer (17%) than in the winter (48%) and in the spring (48%).

Casein concentration, with the exception of γ-CN, showed the lowest values in the summer season and the greatest values in the winter; intermediate values were observed in spring (Table 4). The concentration of α_S-CN in milk during summer was 22.6% lower ($P = 0.0001$) than in the winter and was 16% lower ($P = 0.009$) than in the spring. Also the concentration of

Table 3. Milk characteristics observed in winter, spring, and summer season

Item	Winter	Spring	Summer	SEM[1]	P-value[2]
TS, %	12.58**	12.34**	11.91	0.1273	0.006
SNF, %	9.00**	8.90	8.75	0.0658	0.040
Fat, %	3.80**	3.61**	3.20	0.1141	0.005
Protein, %	3.50**	3.40*	3.29	0.0508	0.020
Lactose, %	5.10	5.13	5.15	0.0380	NS
SCC, log (n·mL^{-1})	4.86*	4.84*	5.18	0.1082	0.067
RCT,[3] min	14.10	12.80*	18.28	1.0835	0.087
k_{20},[4] min	7.87	7.03	8.40	0.6867	NS
a_{30},[5] mm	35.93*	33.60*	21.98	2.9301	0.098

[1]Highest SEM.

[2]P-value of the season effect.

[3]RCT = rennet coagulation time.

[4]k_{20} = curd firming rate.

[5]a_{30} = curd firmness.

Asterisks indicate significant differences from the value observed in summer season (*$P < 0.10$; **$P < 0.05$).

Table 4. Casein concentration in milk samples collected in winter, spring, and summer season

Item	Winter	Spring	Summer	SEM[1]	*P* value[2]
Total CN					
g·L^{-1}	27.50**	24.84*	22.65	1.0715	0.0018
g per 100 g of protein	76.67**	76.55**	74.29	0.9747	0.0400
α_S-CN					
g·L^{-1}	12.80**	11.81**	9.91	0.5489	0.0004
g per 100 g of CN	46.35*	47.27*	43.65	0.8834	0.0058
β-CN					
g·L^{-1}	9.78**	8.54	7.89	0.4098	0.0013
g per 100 g of CN	35.85	34.69	34.78	0.9086	NS
κ-CN					
g·L^{-1}	4.11*	3.73	3.69	0.1999	0.0985
g per 100 g of CN	14.90*	14.97*	16.31	0.5204	0.0190
γ-CN					
g·L^{-1}	0.80**	0.76**	1.21	0.1776	0.0216
g per 100 g of CN	2.90*	3.06*	5.32	0.7312	0.0007

[1]Highest SEM.
[2]*P*-value of the season effect.
Asterisks indicate significant differences from the value observed in summer season (*$P < 0.10$; **$P < 0.05$).

β-CN in milk during summer was 19.3% lower ($P = 0.003$) than in the winter and was 7.7% numerically lower than in the spring. The differences for κ-CN among seasons were more attenuated, with 9.7% lower concentration of κ-CN in milk during summer than in the winter ($P = 0.060$). Conversely, the γ-CN concentration in milk was greater during summer, and in this season was 51% greater than in the winter ($P = 0.030$) and was 59% greater than in the spring. The effect of season on the CN fractions was also significant when the data were processed using the model covaried on SCC, with the exception of k-CN for which the inclusion of SCC was not significant.

A seasonal effect on CN fractions expressed as a percentage of total CN was observed. The percentage of α_S-CN on total CN observed in summer was 5.8% lower ($P = 0.011$) than in the winter and was 7.7% lower ($P = 0.003$) than in the spring. Conversely, the percentage of γ-CN on total CN observed in summer was 83% greater ($P = 0.002$) than in the winter and was 74% greater ($P = 0.009$) than in the spring. Also, the percentage of κ-CN on total CN observed in summer was 9.5% greater than in the winter and in the spring ($P = 0.015$ and $P = 0.033$, respectively). From the statistical analysis, using the model covaried on SCC, the seasonal effect on α_S-CN and γ-CN was still significant; conversely, the seasonal effect on κ-CN was not significant when the data were covaried on SCC. Furthermore, the relative proportion of CN fractions expressed on total protein was 74.3, 76.7, and 76.5% in the summer, winter, and spring, respectively.

A seasonal effect on all whey proteins fractions was observed (Table 5). The concentration of α-LA was greatest in summer and lowest in the winter season. Conversely, β-LG showed the lowest values in summer and the greatest in the winter season. Both these whey proteins showed intermediate values in the spring season. From the statistical analysis, using the model covaried on SCC, the seasonal effect was still significant only for α-LA. The content of IgG was greater in summer than in the winter and spring ($P = 0.049$ and $P = 0.003$, respectively). Similar trend was observed for BSA, with greater values in summer than in the winter and spring ($P = 0.069$ and $P = 0.058$, respectively).

The main results from the PC analysis on milk characteristics have shown that PC1 and PC2 explained only the 22.2 and 19.2% of the total variance, respectively. Figure 3A shows the loading plot of PC1 versus PC2. Total solids, SNF, protein, CN, and a_{30} mainly determined the increase of PC1. The reduction of PC1 was mainly determined by an increase of α-LA, BSA, IgG, γ-CN (expressed both in g·L^{-1} and as a percentage of CN), RCT, and k_{20}. The increase of PC2 was mainly associated with greater κ-CN and α_S-CN concentration, and greater β-CN as percentage of CN. The PC1 separated the milk samples collected in summer from those collected in winter and spring (Figure 3B). Season distribution was mainly obtained along the PC1 axis, which was able to describe only the 22.2% of the total variance. Plotting PC2 against PC1, the summer season was scored in one zone of the plane, and the winter and spring season were on the opposite side.

DISCUSSION

Main Milk Components

Milk yield and milk characteristics may show changes over time as a result of the influence of the seasons on animals. Among the main causes of these variations can

Table 5. Whey protein fraction in milk samples collected in winter, spring, and summer season

Item	Winter	Spring	Summer	SEM[1]	*P*-value[2]
α-LA					
g·L^{-1}	1.73**	1.67**	2.09	0.1204	0.0082
g per100 g of whey protein	20.94**	22.17**	27.29	1.0201	<0.0001
β-LG					
g·L^{-1}	5.85**	5.29	4.73	0.2824	0.0050
g per 100 g of whey protein	69.58**	68.72*	60.76	1.4465	<0.0001
BSA					
g·L^{-1}	0.39*	0.37*	0.49	0.0496	0.0934
g per 100 g of whey protein	4.70**	5.00*	6.22	0.5719	0.0570
IgG					
g·L^{-1}	0.25**	0.17**	0.32	0.0378	0.0091
g per 100 g of whey protein	2.96**	2.28**	4.12	0.4176	0.0030
Lactoferrin					
g·L^{-1}	0.15**	0.14	0.12	0.0096	0.0502
g per 100 g of whey protein	1.82*	1.83*	1.61	0.1190	0.2166

[1]Highest SEM.

[2]*P*-value of the season effect.

Asterisks indicate significant differences from the value observed in summer season (*$P < 0.10$; **$P < 0.05$).

be included dietary factors, in particular the characteristics of feeds, as well as the feeding techniques, housing factors, and the climatic conditions (microclimatic) of the area where the animals are raised. The dietary factors play a major role in some conditions such as cows kept inside and fed silage during winter, and kept on pasture and fed fresh grass during spring-summer. These considerable differences in diets may influence chemical and physical characteristics of milk. In the current study, the cows were raised indoors and the diet was based on the same feeds throughout the seasons. Consequently, it could be hypothesized only a negligible and marginal effect of dietary factors on the changes of milk characteristics observed throughout the seasons.

The climatic issue could be considered the main factor that has affected the changes of milk characteristics throughout the seasons in this study. Po valley, where this study was carried out, is characterized by high temperature and high relative humidity during summertime, causing heat stress in dairy cows, with negative effects on milk characteristics (Bernabucci and Calamari, 1998; Bertocchi et al., 2014) and milk cheese-making properties (Calamari and Mariani, 1998). During the summer in the current study, the mean daily THI was <75 for about 50% of the summer days investigated, whereas it was <72 for only 2 to 3 d. Therefore, according to Bernabucci et al., (2014), cows were subjected to mild-high heat stress during the summer season when our measurements were carried out.

In the current study, the lower concentration of fat and protein in milk were observed in summer and the greater concentration were observed in winter; intermediate values were observed in spring. Reduction of fat and protein concentration and MY as temperature increases has been reported (Casati et al., 1998; Bertocchi et al., 2014). Casati et al. (1998), in a research carried out in Piacenza province (Po valley) studying the season effect on individual MY and characteristics, observed a reduction of milk fat and protein concentration at mean daily temperature above 14°C and at mean daily THI above 55. Bertocchi et al. (2014), in a retrospective study on seasonal variations carried out on bulk milk collected from farms in the Lombardia region (Po valley), observed a negative correlation between THI and fat and protein concentration, and evidenced breakpoints (that represent an inflection point in the relationship between the independent and dependent variable) in the pattern at 50.2 and 65.2 maximum THI, respectively. In the current study, for many days of the spring season the mean daily THI ranged between 62 to 67 and the maximum daily THI ranged from 67 to 76. These values are in the range or above the breakpoints reported before.

Casati et al. (1998) observed that milk fat and protein content decreased in spring and increased in autumn, and suggested that these changes were also related to the photoperiod and variation in MY, as also suggested by Bertocchi et al. (2014). Similarly, Aharoni et al. (2002), in a study carried out on Georgian and Israeli primiparous and Israeli multiparous dairy cows, observed greater fat and protein concentration from October to January, followed by an initial drop in spring and a significant reduction in summer.

In dairy cattle, photoperiod involves a series of hormonal changes, which might affect milk production, DMI, feeding behavior, reproduction, and growth (Dahl et al., 2000). Milk yield is in fact affected by photoperiod and increases as day length increases. Dahl et al. (2000) observed that an increase of daily light length from 12 to 16 h increased the MY of 2.5 kg·animal^{-1}

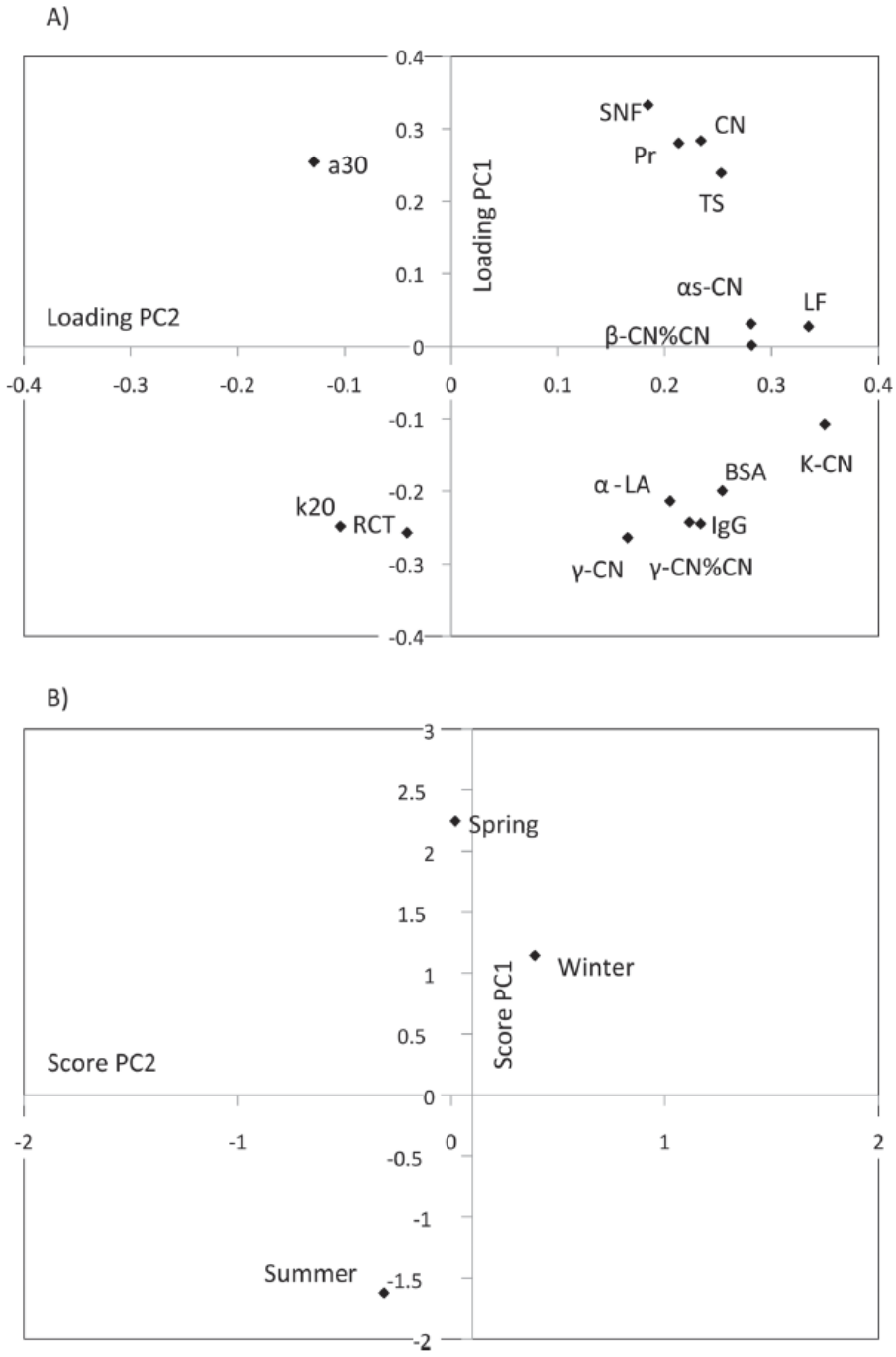

Figure 3. (A) Loading plot of the first 2 principal components (PC1 and PC2). RCT = rennet coagulation time; k_{20} = curd firming rate; a_{30} = curd firmness. (B) Score plot of PC-analyzed samples collected through season using PC1 and PC2. The PC1 and PC2 accounted for 41.4% of the observed variation in the data.

per d. Similarly, Calamari et al. (2002) observed that an increase of daily light length from 10 to 16 h increased the MY of 2.73 kg·animal^{-1} per d, with a concomitant reduction in protein concentration. Auldist et al. (2007) observed in melatonin-treated cows a reduction of prolactin in plasma, a drop in MY of 23%, a reduction of lactose concentration, and an increase of fat, protein, and CN concentration in milk. Therefore, it is plausible that the reduction in milk components recorded in spring might be related to the photoperiod effect and in particular to the increase in MY as a response to the increasing photoperiod, with a consequent dilution effect on protein and fat concentrations. On this regard, Calamari et al. (2002) observed that only the 38% of MY change were responsible for milk protein dilution. In the current study, the average MY in spring was greater than winter (39.7 and. 35.7 L·animal^{-1} per d in spring and winter, respectively). Therefore, it is reasonable that the reduction of fat and protein in spring observed in this study was affected by the photoperiod, in part as a consequence of increased MY with a dilution effect (Bertocchi et al., 2014). Furthermore, the broad decrease in fat and protein content observed during the summer seems mainly related to the negative effect of hot conditions on the synthesis of these milk components.

CN Fractions

In this study, milk protein quantification was performed using SDS-PAGE, a standardized semiquantitative method, that is very useful for comparing different samples on a single gel, but it also has certain limitations, as broad peaks may sometimes result in overlap and degradation products other than the major γ-CN, were not be quantified. γ-Caseins mainly result from postsecretory hydrolysis of β-CN. The current study showed a reduction of CN and a profound change in the CN fractions during the summer season. The main cause of CN reduction during the summer was the reduction in α_S-CN and β-CN, and only the α_S-CN was lower in summer compared with winter and spring both as expressed as absolute (g·L^{-1}) and relative (percentage of total CN) value. These results confirm those obtained by Bernabucci et al. (2002) in a research carried out on dairy cows monitored in spring and summer in a commercial dairy herd in Central Italy, in this case the concentration of protein fractions was measured by electrophoresis on cellulose acetate. Gellrich et al. (2014) recently observed seasonal effect on α-CN and β-CN, with higher β-CN as a percentage of protein in summer season. In this last paper, major milk protein analyses were conducted using a microfluidic electrophoresis and no data were presented on climatic conditions; thus it is not possible to adequately compare the present with the Gellrich et al. study.

The α_S-CN and β-CN contain high numbers of phosphate groups (Schmidt, 1980), and the phosphorylation of CN needs the presence of the γ–phosphate of ATP (Mercier and Gaye, 1983). During heat stress, the reduction in energy intake and the increased maintenance costs cause negative energy balance (Bernabucci et al., 2010). Consequently, we can hypothesize that the lower content of α_S-CN and β-CN in summer milk might be partially due to the reduction in energy and protein availability, which usually occurs during heat stress (Abeni et al., 2007; Bernabucci et al., 2010; Calamari et al., 2013).

Despite marked reductions in nutrient intake, heat-stressed cattle exhibit increased basal insulin levels and stimulated insulin response (Baumgard and Rhoads, 2012). The effect of heat stress on muscle and mammary protein metabolism is unclear because insulin typically stimulates protein synthesis in both tissues (Bernabucci et al., 2010). In heat-stressed cows, the circulating nonesterified fatty acid levels are reduced (Abeni et al., 2007), and the adipose tissue is less sensitive to catabolic signals during heat stress (Baumgard and Rhoads, 2012). It seems that acclimation to heat stress conditions increases a shift toward carbohydrate use (Rhoads et al., 2013), increasing the use of glucose as energy source, decreasing the oxidation of NEFA, and the plasma glucose concentration tended to be lower (Abeni et al., 2007). In addition to lipid and glucose metabolism, protein metabolism is also altered under heat stress conditions (Bernabucci et al., 2010). These metabolic alterations occur coincident with increased circulating basal and stimulated plasma insulin concentrations (Rhoads et al., 2013), influencing MY and milk protein synthesis, in particular with CN fractions more phosphorylated and probably more influenced by energy status.

Recently, Han et al. (2011) observed in vitro study on mammary epithelial cells that heat stress decreased the mRNA level of milk proteins, in particular α-CN and β-CN, and suggested that heat shock induced the stress responses of bovine mammary epithelial cells, with a strong effect on the secretion of protein and fat concentration in milk, and increased the tolerance of the cell itself. Thus, heat stress has direct (tissue hyperthermia) and indirect (reduced feed intake) effects, both of which may affect milk protein synthesis in the mammary gland. Furthermore, the metabolic alterations triggered by hot conditions are also implied.

In the current study, κ-CN decreased in summer when expressed as an absolute value (g·L^{-1}); conversely, the relative percentage of κ-CN was greater in summer. These results confirm those obtained by Bernabucci

et al. (2002). In contrast, Kroeker et al. (1985), in a research carried out analyzing by urea PAGE CN fractions throughout the year, did not observe a definite seasonal trend for the relative percentage of CN fractions. Those authors observed that the relative proportions of CN (β-CN and κ-CN) were more affected by SCC in the milk. In particular, the relative percentage of κ-CN increased with increasing SCC; conversely, the relative percentage of β-CN decreased dramatically with increasing SCC. In the present study, the relative proportion of k-CN did not differ significantly in summer compared with the other seasons when the data were covaried on SCC, and differences between season for α_s-CN and γ-CN were still significant when SCC was included as covariate into the model. Taking into account all results, it could be hypothesized that the synthesis of α_S-CN and β-CN in the mammary gland are more negatively affected by hot conditions, and that the increased percentage of κ-CN is mainly a consequence of a greater reduction of α_S-CN and β-CN synthesis and, in a lesser extent, of a greater breakdown of β-CN. Moreover, differences in results of CN relative percentage may in part be due to the separation method used.

Whey and Serum Protein Fractions

In this study the concentrations of α-LA were greater during summer season compared with the other seasons. The literature contains little information on changes of whey proteins during the hot summer months. Bernabucci et al. (2002) did not observe any difference for α-LA and β-LG between seasons. Gellrich et al. (2014), in a research carried out in Germany studying the effect of season, observed greater α-LA concentration in summer compared with the fall season. Similarly, Brodziak et al. (2012) found a significantly higher concentration of α-LA and a higher ratio of α-CN:β-CN in milk of cows from the spring-summer season, in comparison with the fall-winter period. In heat-stressed cows, the reduction of energy intake and the increased maintenance costs causes often negative energy balance (Bernabucci et al., 2010), and the increased α-LA observed in hot conditions does not seem related to the impairment of energy balance.

The insulin infusion plus glucose to minimize blood glucose changes increased MY and the proportions of α-LA and β-LG (Mackle et al., 1999). Glucagon infusion decreased the synthesis of α-LA as well as of β-LG, with a reduction in the relative percentage of α-LA but not of β-LG. Similarly, atropine injection, which reduces plasma amino acids and plasma insulin concentration, tends to decrease the concentrations of α-LA in milk (Auldist et al., 2003). In heat-stressed cows, the basal insulin is increased, and it seems involved in the increased α-LA observed in milk. However, also considering that bST injection preferentially increases α-LA synthesis (Eppard et al., 1985), the endocrinological changes commonly observed in heat-stressed cows are controversial on the expected α-LA changes in milk.

A greater BSA concentration in milk was observed in this study during summer compared with the other seasons. Serum albumin enters in milk via the paracellular route throughout loosening tight junction or in association with neutrophil diapedesis (or both), not necessarily under damage of lactocytes. An increase of milk BSA was observed during intramammary infection (Shuster et al., 1991). In the current study, SCC were low and only slight greater values in summer compared with the other seasons was observed. Furthermore, the greater BSA in summer was still significant when the data were covaried on SCC. Then, we can exclude mastitis as the possible and main cause of greater BSA in milk during summer season.

Lu et al. (2003), in research carried out in Holstein cows under humid tropical summer climates, observed greater BSA in milk of summer cows, at the same time the SCC remained below 5×10^5 cells per mL, but *N*-acetyl-glucosaminidase increased linearly with the progress of summer season (Lu et al., 2003). *N*-Acetyl-glucosaminidase activity in milk is considered as a direct indicator of secretory cell damage. Steady increase of N-acetyl-glucosaminidase activity in milk could mean a gradual deterioration of mammary epithelial barrier under the summer climates of the Lu et al. (2003) study. Those authors concluded that gradual regression of mammary gland occurred along the humid tropical summer season. The increased BSA observed in our study could be due also to the alteration of the mammary epithelial barrier.

Milk Coagulation Properties

The milk produced during summer period by cows raised in unfavorable climatic conditions, shows a worsening in the main cheesemaking characteristics, in particular a worsening of MCP (Calamari and Mariani, 1998; Malacarne et al., 2005). In the current study, a higher percentage of milk samples with scarce or very bad reactivity, as well as a lower percentage of milk samples having good coagulation properties was observed in summer.

The results obtained with the PC analysis have shown that plotting PC1 and PC2, the summer season was confined in one zone of the plane, and the winter and spring seasons were on the opposite side. The discrimination of summer was mainly related to the milk solids (negative relationship with total solids,

SNF, protein, and CN concentration), together with the MCP (positive relationship with RCT and curd firming rate, and negative relationship with curd firmness). These results confirm the relationship between CN content and MCP (Calamari and Mariani, 1998; Bertoni et al., 2001, 2005). Furthermore, the discrimination of summer season was also related to the effect of BSA and γ-CN (positive relationship), but the effect of SCC was negligible and did not contribute to the discrimination among seasons. These results indicate that the increase of these protein fractions during summer was not related to the only slight increase of SCC and that the worsening of MCP during summer was also a consequence of these milk protein fraction changes.

In this study, the κ-CN concentration was lower in summer milk, but the relative percentage of this CN fraction slightly increased in the summer season. Better MCP was observed in milk with higher κ-CN concentration (Marziali and Ng-Kwai-Hang, 1986). Wedholm et al. (2006) observed that poorly and noncoagulating samples were associated with a low concentration of κ-CN and a low relative proportion of κ-CN. Mariani et al. (1998) observed that a greater availability of κ-CN per unit of α_{S1}-CN would lead to a configuration such as to be more favorable from the viewpoint of coagulation properties: improved enzymatic reactivity of the micelles and increased capacity of aggregation of para-CN. It should be noted, however, that small differences in the fraction of κ-CN could induce significant changes in the attitude to clot: the 1% more or less of κ-CN implies a 20% change in the diameter of the micelles. Higher values of κ-CN micelles are associated with smaller (Laurent et al., 1992) and therefore more suitable for the coagulation. It should be also considered that an increase in either total κ-CN concentration or relative percentage of κ-CN (percentage of total CN) in general decreases overall levels or relative percentage of α_{S1}-CN and α_{S2}-CN, affecting milk coagulation through the effect on pH (Bittante et al., 2012). All these findings seem to indicate that in the summer season the concentration of κ-CN in milk, and in particular its relative proportion, does not appear among the main and direct factors that negatively affect the MCP.

In the current study, a lower α_S-CN and β-CN concentration was observed in summer milk, and only the former was also lower as a relative proportion. Contents and relative percentage of α_S-CN and β-CN, due to their high sensitivity toward precipitation by calcium ions, strongly influence milk coagulation properties (Schmidt, 1980). The effects of these fractions on the MCP are controversial. Remeuf and Hurtaud (1991) observed that high values of β-CN and low of α_{S1}-CN are favorable for coagulation, in particular for clotting time. According to Pearse and Mackinlay (1989), β-CN play an essential role in syneresis. Grandison et al. (1984) found positive relationships between coagulum strength and α_S-CN and β-CN concentrations. Conversely, Jõudu et al. (2008) observed that milk formed a firmer curd when the relative proportion of a_{S1}-CN and β-CN in total CN was lower.

The α_S-CN and β-CN, rich in phosphate groups, are the 2 acidic components of the CN micelles (Schmidt, 1980). Thus, the lower contents of α_S-CN and β-CN of milk yielded during the summer might explain the higher milk pH and the lower milk titratable acidity commonly registered during the hot summer months (Calamari and Mariani, 1998). The clotting time is heavily influenced by pH and titratable acidity of milk, and also curd firmness is greatly affected by milk acidity (Bittante et al., 2012). Our results seem to indicate that the worsening of MCP observed in summer could have been promoted by the reduction of the concentration and relative proportion of α_S-CN and β-CN, involving consequences on pH and titratable acidity.

CONCLUSIONS

The reduction of milk protein concentration during hot conditions in summer season seems to be mainly attributable to the reduction in CN concentration. The α_S-CN and β-CN are the CN fractions most sensitive to hot conditions. The CN composition changes throughout seasons, with a reduction of the proportion of α_S-CN and β-CN and an increase of the proportion of κ-CN and γ-CN during summer. The worsening of the MCP observed during summer seems mainly attributable to the reduction of protein concentration and changes in milk protein fractions. Besides the increase of the protein fractions related to the increased proteolysis during the hot season, a change also occurred throughout the seasons in the proportion of protein fractions obtained with electrophoresis and expressed as a percentage of total protein (N × 6.38) concentration in milk. This increase of undefined proteins observed only during summer needs to be deeply investigated for a better understanding of the relationships between milk protein fractions and MCP in the summer season.

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REFERENCES

Abeni, F., L. Calamari, and L. Stefanini. 2007. Metabolic conditions of lactating Friesian cows during hot season in Po valley. 1. Blood indicators of heat stress. Int. J. Biometeorol. 52:87–96.

Aharoni, Y., O. Ravagnolo, and I. Misztal. 2002. Comparison of lactational responses of dairy cows in Georgia and Israel to heat load and photoperiod. Anim. Sci. 75:469–476.

Auldist, M. J., C. M. Menzies, and C. G. Prosser. 2003. Effects of atropine on plasma amino acid profiles and on the synthesis of individual milk proteins in dairy cows fed with pasture. J. Dairy Res. 70:373–378.

Auldist, M. J., S. A. Turner, C. D. McMahon, and C. G. Prosser. 2007. Effects of melatonin on the yield and composition of milk from grazing dairy cows in New Zealand. J. Dairy Res. 74:52–57.

Baumgard, L. H., and R. P. Rhoads. 2012. Ruminant nutrition symposium: ruminant production and metabolic responses to heat stress. J. Anim. Sci. 90:1855–1865.

Bernabucci, U., S. Biffani, L. Buggiotti, A. Vitali, N. Lacetera, and A. Nardone. 2014. The effects of heat stress in Italian Holstein dairy cattle. J. Dairy Sci. 97:471–486.

Bernabucci, U., and L. Calamari. 1998. Effects of heat stress on bovine milk yield and composition. Zootech. Nutr. Anim. 24:247–258.

Bernabucci, U., N. Lacetera, L. H. Baumgard, R. P. Rhoads, B. Ronchi, and A. Nardone. 2010. Metabolic and hormonal acclimation to heat stress in domesticated ruminants. Animal 4:1167–1183.

Bernabucci, U., N. Lacetera, B. Ronchi, and A. Nardone. 2002. Effects of hot season on milk protein fractions in dairy cows. Anim. Res. 51:25–33.

Bertocchi, L., A. Vitali, N. Lacetera, A. Nardone, G. Varisco, and U. Bernabucci. 2014. Seasonal variations in the composition of Holstein cow's milk and temperature-humidity index relationship. Animal 8:667–674.

Bertoni, G., L. Calamari, G. Maianti, and B. Battistotti. 2005. Milk for protected denomination of origin (PDO) cheeses: I. The main required features. Pages 217–228 in Indicators of milk and beef quality. J. F. Hocquette and S. Gigli, ed. EAAP Pubblication No. 112, Wageningen Academic Publishers, Wageningen, the Netherlands.

Bertoni, G., L. Calamari, and M. G. Maianti. 2001. Producing specific milk for speciality cheeses. Proc. Nutr. Soc. 60:231–246.

Bittante, G., M. Penasa, and A. Cecchinato. 2012. Invited review: Genetics and modeling of milk coagulation properties. J. Dairy Sci. 95:6843–6870.

Brodziak, A., J. Barlowska, J. Kròl, and Z. Litwińczuk. 2012. Effect of breed and feeding system on content of selected whey proteins in cow's milk in spring-summer and autumn-winter seasons. Ann. Anim. Sci. 12:261–269.

Calamari, L., M. G. Maianti, and A. Azzoni. 2002. Effetti del fotoperiodo sulla produzione e su alcune caratteristiche del latte di bovine Frisone. Sci. Tecn. Latt. Cas. 53:189–206.

Calamari, L., and P. Mariani. 1998. Effects of the hot environment conditions on the main milk cheesemaking properties. Zootec. Nutr. Anim. 24:259–271.

Calamari, L., F. Petrera, L. Stefanini, and F. Abeni. 2013. Effects of different feeding time and frequency on metabolic conditions and milk production in heat stressed dairy cows. Int. J. Biometeorol. 57:785–796.

Casati, M. R., V. Cappa, L. Calamari, F. Calegari, and G. Folli. 1998. Influenza delle stagioni sulla produzione e su talune caratteristiche del latte bovino. Sci. Tecn. Latt. Cas. 49:7–25.

Dahl, G. E., B. A. Buchanan, and H. A. Tucker. 2000. Photoperiodic effects on dairy cattle: A review. J. Dairy Sci. 83:885–893.

Eppard, P. J., D. E. Bauman, J. Bitman, D. L. Wood, R. M. Akers, and W. A. House. 1985. Effect of dose of bovine growth hormone on milk composition: α-Lactalbumin, fatty acids, and mineral elements. J. Dairy Sci. 68:3047–3054.

Gellrich, K., H. H. D. Meyer, and S. Wiedemann. 2014. Composition of major proteins in cow milk differing in mean protein concentration during the first 155 days of lactation and the influence of season as well as short-term restricted feeding in early and mid-lactation. Czech J. Anim. Sci. 59:97–106.

Giacinti, G., L. Basiricò, B. Ronchi, and U. Bernabucci. 2013. Lactoferrin concentration in buffalo milk. Ital. J. Anim. Sci. 12:139–143.

Grandison, A. S., G. D. Ford, D. Millard, and A. J. Owen. 1984. Chemical composition and coagulating properties of renneted milks from cows during early lactation. J. Dairy Res. 51:407–416.

Hahn, G. L., and T. L. Mader. 1997. Heat waves in relation to thermoregulation, feeding behavior and mortality of feedlot cattle. Pages 563–571 in Proc. 5th Int. Livest. Environ. Symp. (Vol. I). ASAE SP-01–97. American Society Agricultural Engineers, St. Joseph, MI.

Han, H., W. JiaQi, L. FaDi, B. DengPan, Z. LinYun, and C. RuiLian. 2011. Responses of cultured bovine mammary epithelial cells to heat stress. J. Agric. Biotechnol. 19:287–293.

Ingraham, R. H., R. W. Stanley, and W. C. Wagner. 1979. Seasonal effects of tropical climate on shaded and nonshaded cows as measured by rectal temperature, adrenal cortex hormones, thyroid hormone, and milk production. Am. J. Vet. Res. 40:1792–1797.

Intergovernmental Panel on Climate Change. 2007. The Intergovernmental Panel on Climate Change 4th Assessment Report (IPCC: AR4). http://www.ipcc.ch/publications_and_data/publications_and_data_reports.htm#2.

Jõudu, I., M. Henno, T. Kaart, T. Püssa, and O. Kärt. 2008. The effect of milk protein contents on the rennet coagulation properties of milk from individual dairy cows. Int. Dairy J. 18:964–967.

Kadzere, C. T., M. R. Murphy, N. Silanikove, and E. Maltz. 2002. Heat stress in lactating dairy cows: A review. Livest. Prod. Sci. 77:59–91.

Kroeker, E. M., F. Ng-Kway-Hang, J. F. Hayes, and J. E. Moxley. 1985. Effects of environmental and milk protein polymorphism on composition of casein fraction in bovine milk. J. Dairy Sci. 68:1752–1757.

Laurent, F., D. Coomans, J. N. Gardeur, and B. Vignon. 1992. Composition azotée et caractéristiques technologiques du lait de vache en relation avec la nature et le niveau d'apport de l'aliment concentré. Lait 72:175–183.

Lu, C. H., C. J. Chang, P. N. Lee, C. P. Wu, M. T. Chen, and X. Zhao. 2003. Functional characterization of mammary gland of Holstein cows under humid tropical summer climates. Asian-australas. J. Anim. Sci. 16:988–995.

Mackle, T. R., A. M. Bryant, S. F. Petch, J. P. Hill, and M. J. Auldist. 1999. Nutritional influences on the composition of milk from cows of different protein phenotypes in New Zealand. J. Dairy Sci. 82:172–180.

Malacarne, M., S. Fieni, F. Tosi, P. Franceschi, P. Formaggioni, and A. Summer. 2005. Seasonal variations of the rennet-coagulation properties of herd milks in Parmigiano-Reggiano cheese manufacture: Comparison between Italian Friesian and Italian Brown cattle breeds. Ital. J. Anim. Sci. 4(Suppl. 2):242–244.

Mariani, P., A. Summer, M. Franchetti, P. Vecchia, and E. Fossa. 1998. Ripartizione percentuale delle caseine in latti di massa delle vacche di razza Frisona, Bruna, Reggiana e Modenese. Sci. Tecn. Latt. Cas. 49:181–192.

Marziali, A. S., and K. F. Ng-Kwai-Hang. 1986. Effects of milk composition and genetic polymorphism on coagulation properties of milk. J. Dairy Sci. 69:1793–1798.

Mercier, J. C., and P. Gaye. 1983. Milk protein synthesis. Pages 177–230 in Biochemistry of Lactation. T. B. Mepham, ed. Elsevier Science Publisher B.V., Amsterdam, the Netherlands.

Nardone, A., B. Ronchi, N. Lacetera, M. S. Ranieri, and U. Bernabucci. 2010. Effects of climate changes on animal production and sustainability of livestock systems. Livest. Sci. 130:57–69.

Pearse, M. J., and A. G. Mackinlay. 1989. Biochemical aspect of syneresis: A review. J. Dairy Sci. 72:1401–1407.

Remeuf, F., and C. Hurtaud. 1991. [Liaisons entre les caracteristiques physico-chimiques des laits et leur aptitude fromagere]. Pages 15–26 in Proc. Qualité des laits a la production et aptitude fromagère. M. Journet and A. Holden, ed. INRA-ENSA, Rennes, France.

Rhoads, R. P., L. H. Baumgard, J. K. Suagee, and S. R. Sanders. 2013. Nutritional interventions to alleviate the negative consequences of heat stress. Adv. Nutr. 4:267–276.
Schmidt, D. G. 1980. Colloidal aspects of casein. Neth. Milk Dairy J. 34:42–64.
Segnalini, M., U. Bernabucci, A. Vitali, A. Nardone, and N. Lacetera. 2013. Temperature humidity index scenarios in the Mediterranean basin. Int. J. Biometeorol. 57:451–458.
Shuster, D. E., R. J. Harmon, J. A. Jackson, and R. W. Hemken. 1991. Suppression of milk production during endotoxininduced mastitis. J. Dairy Sci. 74:3763–3774.
Wedholm, A., L. B. Larsen, H. Lindmark-Mansson, A. H. Karlsson, and A. Andren. 2006. Effect of protein composition on the cheese-making properties of milk from individual dairy cows. J. Dairy Sci. 89:3296–3305.