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Conjugated linolenic acid (CLnA) isomers as new bioactive lipid compounds in ruminant-derived food products. A review

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ABSTRACT. Conjugated linolenic acid (CLnA) isomers refer to a group of positional and geometric isomers of the omega-3 essential fatty acid – α-linolenic acid (*cis-9,cis-12,cis-15* C18:3; ALA). CLnA isomers can be either *cis*- and/or *trans*- and their double bonds are separated by a single bond. Food products from ruminants and some plant products (e.g., pomegranate or bitter melon seeds) are the major sources of CLnA isomers for humans. CLnA isomers in ruminants arise as a result of bacterial isomerization of ALA in the rumen. The concentration of CLnA isomers in seed oils is higher than in milk and edible parts of ruminant carcass. The CLnA isomers from the plant sources are in the form of conjugated trienes, whereas those in ruminant products are of conjugated diene type. Some plant seed oils are the richest natural sources of CLnA isomers. So searching for methods of increasing the CLnA isomer content in food of animal origin not exhibiting negative effects on animal welfare and physiology is very important for researchers. A presence of long-chain and very long-chain conjugated unsaturated fatty acids was also confirmed in some ruminant tissues. Clinical studies documented that health-promoting properties have been attributed to CLnA isomers. It was also evidenced that animal diet may influence the CLnA synthesis. The proper identification of geometric and positional isomers of conjugated unsaturated fatty acids in biological samples is a great analytical challenge. Therefore, silver-ion high-performance liquid chromatography with photodiode detection and capillary gas chromatography (GC) offer the best analysis of these isomers with complementary identification by GC-mass spectrometry.

Introduction

Animal-derived food products are considered for their both positive and negative nutritional attributes. Meat, milk and dairy products are major sources of many bioactive compounds, e.g., proteins, lipids, vitamins, indispensable amino acids or essential elements (like Se, Zn, Cu, Fe, I, Mn, Cr, Co, Ni or Mo). These bioactive compounds, however, may be also associated with negative nutritional profiles attributed to high concentrations of saturated fatty acids (SFA), n-6 polyunsaturated fatty acids (n-6 PUFA), cholesterol, sodium as well as high caloric contents (Decker et al., 2010).

However, some unique compounds like lipids, fatty acids (FA), complex carbohydrates and peptide sequences encrypted within milk proteins and exerting beneficial activities are specific only for these foods (Mills et al., 2011). So, many researchers focus on ways to increase contents of health-promoting bioactive compounds (like n-3 PUFA, vitamins or Se-species) in humans (Manso et al., 2016). However, the enhancement of edible parts of ruminant carcass in health-promoting unsaturated fatty acids (UFA), especially n-3 PUFA, is highly dependent on rumen biohydrogenation (Petersen, 2014).

Therefore, the study of rumen fat metabolism is very important to understand factors affecting the FA profile in human food products derived from ruminants. Numerous studies documented that food products of ruminant origin are naturally rich in vaccenic acid (*trans-11* C18:1; VA) and conjugated linoleic acid (CLA) isomers, particularly *cis-9,trans-11* C18:2 (*c-9,t-11* C18:2) (Buccioni et al., 2012). Fortunately, concentrations of these health-promoting fatty acids in edible parts of ruminant carcasses are to some extent affected by the animal diet (Buccioni et al., 2012). Recently, much attention is also paid to the conjugated linolenic acid (CLnA) isomers, due to promising results of studies referring to their very important physiological properties. Therefore, the biosynthesis and metabolism of CLnA isomers in ruminants were investigated and the strategies of enrichment of meat and milk with these conjugated FA are commonly known (Wąsowska et al., 2006; Modaresi et al., 2011; Razzaghi et al., 2015).

The main aim of this review was to summarize the latest findings about ruminal biohydrogenation of α-linolenic acid (*c-9,c-12,c-15* C18:3; ALA), putting special emphasis on biosynthesis and metabolism of its conjugated isomers. Strategies to increase CLnA content in meat and milk through modification of animal feeding as well as the most important aspects of analysis of these isomers are also described.

Conjugated fatty acids – chemical structure, biosynthesis and sources

Structure of majority of polyunsaturated fatty acids (PUFA) is characterized by a methylene interrupted double bonds in carbon chains. If this methylene group is removed from between these two bonds, the conjugated structure is created and the resulting fatty acid is called conjugated fatty acid (CFA). This is a general term for a group of both geometric and positional isomers of PUFA, which may be formed into dienes, trienes or tetraenes (Yuan et al., 2014). This unique structure impinges on their specific chemical properties and physiological activity. The best-known group of CFA is a group of linoleic acid isomers *(c-9,c-12* C18:2; LA) called conjugated linoleic acids (CLA). Theoretically, due to the difference in geometric (*cis* or *trans*) and positional configurations as well as various substituents, the existence of 56 CLA isomers is possible (Roach et al., 2002).

CLA isomers in ruminants result from biotransformation of LA and ALA. They are subjected to the isomerization and hydrogenation by anaerobic microorganisms colonizing the rumen (Ogawa et al., 2005). The most abundant CLA isomer is *c-9,t-11* C18:2 (rumenic acid; RA), which constitutes about 90% of total pool of CLA isomers. Other possible positional and geometric CLA isomers are *c,c*; *c,t/t,c* and *t,t.* RA, an intermediate of reduction of LA to stearic acid (C18:0), is also efficiently formed as an effect of action of Δ9-desaturase on VA, occurring both in muscles and mammary gland (Tanaka, 2005). About 78% of the total pool of RA in cow's milk fat is created during endogenous synthesis (Corl et al., 2001). Part of RA, which is not hydrogenated to VA or C18:0 in the rumen, is absorbed from the gastrointestinal tract, and together with blood is transported into mammary gland. However, the share of this pathway in RA synthesis is negligible (Białek and Tokarz, 2013).

As a result of the action of microorganisms inhabiting rumen, other minor CLA isomers are also formed (e.g., *t-10,c-12* C18:2). This process is the main source of CLA isomers in milk of polygastric animals (Pariza et al., 1999). In mammals due to the lack of Δ12-desaturase, *t-10* C18:2 cannot be a substrate of endogenous biosynthesis of *t-10,c-12* C18:2.

Investigation of CLA isomers biological features began in 1980s when Pariza et al. (1979) found in bovine meat (both raw and fried) a previously unknown compound with mutagenic inhibitory activity which was confirmed to be the effect of CLA isomers (Ha et al., 1987). Thereafter, in various animal models (e.g., rats, mice and ruminants) and in humans it was proved that some CLA isomers can exert positive effect on different pathological conditions, such as atherosclerosis, diabetes, obesity and different types of cancer (Park and Pariza, 2007; Wallace et al., 2007; Badinga et al., 2016).

Nevertheless, CLA isomers are not the only one group of bioactive CFA. Recently, more attention is paid to ALA isomers with conjugated double bonds (Koba et al., 2007a). In contrast to CLA isomers present in abundance in ruminant-derived products (meat, milk, dairy products), conjugated linolenic acids (CLnA) are present mainly in plants and they are usually most prevalent among all fatty acids (>70%) (de Carvalho et al., 2010). Each plant has a specific enzyme – conjugase, which converts ALA into particular CLnA isomer, which is then accumulated in seed tissues (Koba et al., 2007a): α-eleostearic acid (*c-9,t-11,t-13* C18:3) in bitter melon (*Momordica charantia*), punicic acid (*c-9, t-11,c-13* C18:3) in pomegranate (*Punica granatum*), catalpic acid (*t-9,t-11,c-13* C18:3) in catalpa (*Catalpa ovata*), calendic acid (*t-8,t-10,c-12* C18:3) in pot marigold (*Callendula officinalis*), jacaric acid (*c-8,t-10,c-12* C18:3) in jacaranda (*Jacaranda mimosifolia*) (Białek et al., 2014a). Divergent isomers of CLnA are present also in animal products, but in lower concentrations (de Carvalho et al., 2010).

CLnA isomers from plants are conjugated trienes, while these from ruminants are conjugated dienes (partially conjugated). Differences between isomers of CLnA from plant and animal sources are presented in Figure 1.

Biohydrogenation of ALA – new insight in biosynthesis of CLnA isomers in ruminants

Main pathways of ALA biohydrogenation

The main pathway of ALA biohydrogenation is initiated by the anaerobic microbial population (*Butyrivibrio fibrisolvens, Butyrivibrio proteoclasticus, Propionibacterium acnes*) (Buccioni et al., 2012) which rapidly isomerizes ALA *via* activity of Δ12-*c*,Δ11-*t*-isomerase to the *c-9,t-11,c-15* C18:3 (rumelenic acid; RLnA) (Harfoot and Hazlewood, 1997). Next, the *c-9* double bond is hydrogenated to produce a non-conjugated dienoic fatty acid (*t-11,c-15* C18:2), followed by hydrogenation of the *t-11/c-15* double bond to produce a monoenoic fatty acids. These C18:1 isomers are further hydrogenated to C18:0 (Lee and Jenkins, 2011).

The results of many research (Shingfield et al., 2010a) showed the complexity of ALA biohydrogenation in the rumen. Alternative pathways resulting in plenty of C18:3 isomers differ in double bond geometry and location, including their conjugated configuration. Some authors predict that fourteen intermediates of C18:3 are possible (Lee and Jenkins, 2011) (Figure 2).

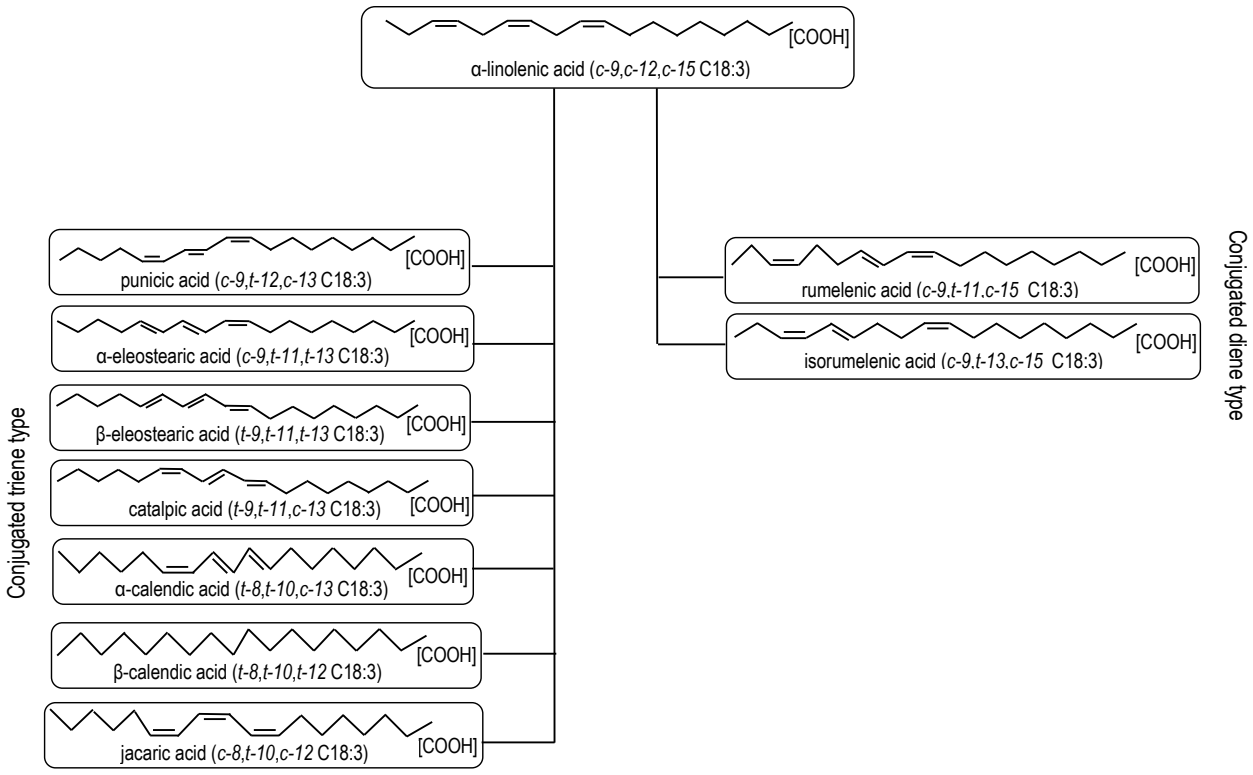

Figure 1. Chemical structure of conjugated linolenic acid (CLnA) isomers of different origin (own compilation based on: Destaillats et al., 2005a; Koba et al., 2007a; Hennessy et al., 2011)

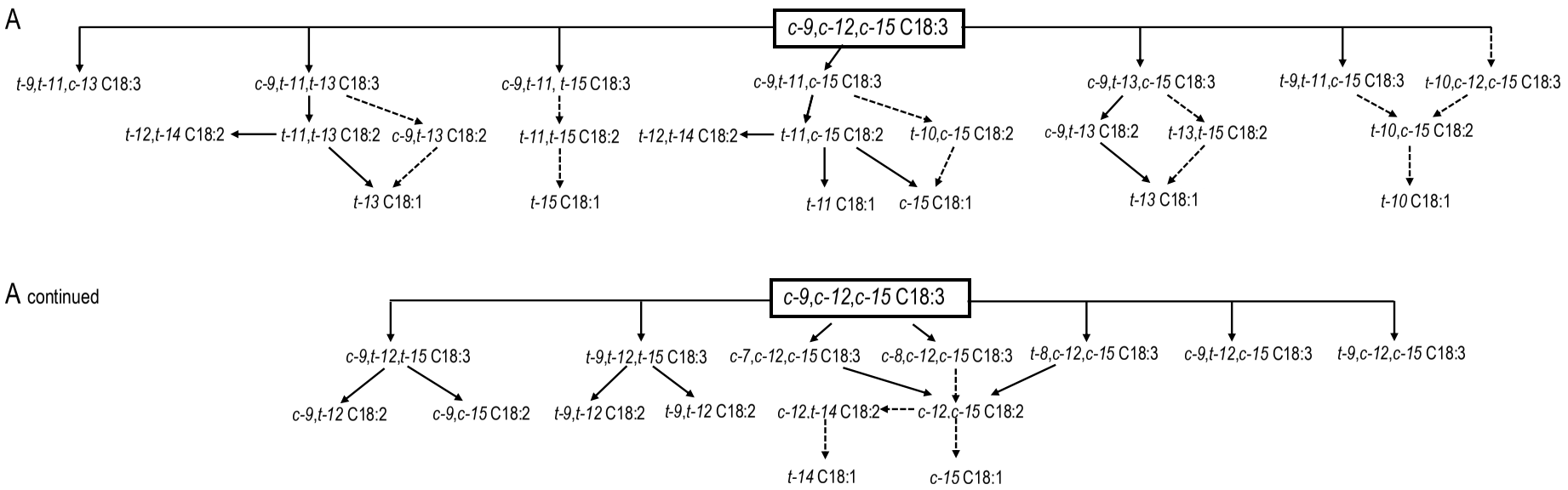

Figure 2. Summary of known and presumptive *c-9,c-12,c-15* C18:3 (α-linolenic acid) biohydrogenation pathways in rumen (own compilation based on: Destaillats et al., 2005a; Bessa et al., 2007; Gómez-Cortés et al., 2009; Lee and Jenkins, 2011; Modaresi et al., 2011; Buccioni et al., 2012; Alves et al., 2014; Petersen et al., 2014; Saliba et al., 2014; Mapiye et al., 2015; Honkanen et al., 2016)
Dashed lines indicate presumptive pathways of α-linolenic acid biohydrogenation. Bottom part of figure (A continued) is continuation of bacterial isomerization and biohydrogenation of α-linolenic acid

Other pathways of 18:3 biohydrogenation include formation of *t-10,c-12,c-15* C18:3 (Griinari and Bauman, 1999) and *c-9,t-13,c-15* C18:3 (isorumelenic acid) isomers (Destaillats et al., 2005b). Rumelenic and isorumelenic acids were detected in meat and milk fat from ruminant species; the *c-9,t-11,c-15* C18:3 was found in both muscle and milk lipids, whereas the *c-9,t-13,c-15* C18:3 was detected only in muscles (Plourde et al., 2007). In cattle duodenal digesta were detected three non-conjugated isomers: *c-9,t-12,c-15* C18:3, *c-9,t-12,t-15* C18:3 and *t-9,t-12,t-15* C18:3 (Loor et al., 2004). Subsequently *c-9,t-11,t-15* C18:3 was characterized in ovine milk fat (Gómez-Cortés et al., 2009), which may originate from the isomerization of the *c-15* bond in rumelenic acid yielded from biohydrogenation. Incubation of ALA with the rumen content resulted in the formation of uncharacterized so far isomers (like *t-9,t-11,c-15* C18:3, *t-9,t-11,c-13* C18:3, *c-7,c-12,c-15* C18:3, *c-8,c-12,c-15* C18:3 and *t-8,c-12,c-15* C18:3), which were formed by different mechanisms (Honkanen et al., 2016). The synthesis of *t-9,t-11,c-13* C18:3 involves the exchange of hydrogen atom with water while *c-7, c-12,c-15* C18:3, *c-8,c-12,c-15* C18:3 and *t-8, c-12,c-15* C18:3 isomers are formed directly from ALA, without hydrogen atom exchange and initial conversion to rumelenic acid (Honkanen et al., 2016). Appearance of these intermediates confirms that ALA isomerization also involves migration of *c-9* double bonds.

Conversion of *c-9,t-11,c-15* C18:3 to *t-11, c-15* C18:2 (vaccelenic acid) and further hydrogenation of the *t-11/c-15* C18:1 allows to make the assumption that CLA are not formed through ALA biohydrogenation (Honkanen et al., 2016). Multitude of C18:3 isomers arising from the ALA isomerization in the rumen through the reduction (hydrogenation) reactions yielded also in a great number of C18:2 isomers, both with non-conjugated and conjugated bonds. Destaillats et al. (2005b) confirmed the synthesis of two CLA isomers: *c-9,t-11* C18:2 and *t-13,c-15* C18:2 from ALA biohydrogenation. More recently, CLA isomer synthesis from ALA acid was proven by Lee and Jenkins (2011) by isotopic ^{13}C enrichment of 8 CLA isomers. It was found that for the *c-9,c-11* C18:2; *t-8,t-10* C18:2 and *c-10,c-12* C18:2 isomers more than half of carbon atoms in the chain were provided by ^{13}C-labeled ALA.

Putative pathways of ALA biohydrogenation

Vaccelenic acid is the main C18:2 isomer formed from rumelenic acid, which may act as precursor for CLnA synthesis *via* Δ9-desaturase activity (Dugan et al., 2011). Recently, the existence of so called '*trans-10'* and '*trans-13'* shifted pathways of rumen biohydrogenation was also established (Alves and Bessa, 2014; Saliba et al., 2014). First of these proposed pathways consists of biotransformation of *t-10,c-12,c-15* C18:3 into the *t-10,c-15* C18:2 isomer and its further reduction to the *t-10* C18:1. This action is introduced when animals are fed ration with high starch content (Leat et al., 1977). Some authors predict also that *t-10,c-15* C18:2 may be derived from rumelenic acid and *t-9,t-11,c-15* C18:3 (Kishino et al., 2009) although other researchers claim that this isomer can be formed only from the C18:2 (Zened et al., 2011). Due to the fact that *t-10,c-15* C18:2 was detected in abomasal and rumen digesta and muscles of lambs fed diet supplemented with ALA, it may be suggested that these metabolic pathways were sources of *t-10,c-15* C18:2 (Alves and Bessa, 2014).

Putative '*trans-13*' biohydrogenation pathway, firstly proposed by Destaillats et al. (2005b) includes initial isomerization of ALA into isorumelenic acid (*c-9,t-13,c-15* C18:3) and its further biohydrogenation to *c-9,t-13* C18:2, *t-13,c-15* C18:2 and *t-13* C18:1 (Saliba et al., 2014). *c-9,t-13* C18:2 isomer, similarly to RA (*c-9,t-11* C18:2), may also be endogenously produced *via* Δ9-desaturation of *t-13* C18:1 in tissues (Loor et al., 2005). Other possible intermediate of this pathway detected in bovine milk fat is *c-9,t-11,t-13* C18:3 (Lerch et al., 2012), which can be converted to *t-11,t-13* C18:2 (Loor et al., 2004). Identification of isorumelenic acid in ruminant milk and meat (Plourde et al., 2007; Rego et al., 2009) as well as the presence of other isomers with *t-13* double bond (Lerch et al., 2012) seem to confirm the existence of '*trans-13*' biohydrogenation pathway.

Increasing content of *t-12,t-14* C18:2, *c-9, t-14* C18:2, *c-12,t-14* C18:2 and *t-14* C18:1 in milk resulted from incorporation of linseed oil or extruded linseeds into ruminants ration (Gómez-Cortés et al., 2009; Saliba et al., 2014) as well as incubation of ALA with rumen digesta (Honkanen et al., 2016) suggested '*trans-14*' to be another alternative pathway of biohydrogenation (Figure 2). *t-12,t-14* C18:2

isomer may be generated by the mechanism of exchanging hydrogen atom with water from geometric isomers of *t-11,t-13* C18:2 or from *t-11,c-15* C18:2 by isomerization (Honkanen et al., 2016).

Deposition of biohydrogenation intermediates of UFA in tissues

The extent of ruminal biohydrogenation as well as pattern of arising intermediates determine the amount of fatty acids absorbed and deposited in animal tissues. LA, ALA and long-chain PUFA are preferentially incorporated into the polar fraction of lipids (Wood et al., 2008) while different isomers of C18:1, C18:2 and C18:3 formed during biohydrogentaion are diversely divided between lipid fractions (Jerónimo et al., 2011). Such acids as vaccenic acid (*t-11* C18:1), rumenic acid (*c-9,t-11* C18:2) as well as vaccelenic (*t-11,c-15* C18:2) and rumelenic acid (*c-9,t-11,c-15* C18:3) were preferentially incorporated into neutral fraction of intramuscular fat of lambs fed linseed oil. Such dependency was also confirmed by Plourde et al. (2006) in tissues of rats fed rumenic acid or equimolar mixture of rumelenic and isorumelenic acids, either as free fatty acids or in triacylglycerols biomolecules. Other work also showed that one of the plant-derived CLnA isomers – punicic acid (*c-9,t-11,c-13* C18:3; PA) was incorporated into the human plasma and red blood cell membranes after 28-day diet supplementation with *Trichosanthes kirilowii* seed kernels containing 3 g of PA per day in the form of triacylglycerols (Yuan et al., 2009a).

Biohydrogenation intermediates of long-chain and very long-chain UFA

Long-chain fatty acids (up to 21 carbon atoms; LCFA) are also intensively metabolized in the rumen. The decreased concentrations of eicosapentaenoic (C20:5n-3; EPA) and docosahexaenoic (C22:6n-3; DHA) acids during incubation with rumen fluid *in vitro* (Wąsowska et al., 2006) confirmed extensive biohydrogenation of these highly-unsaturated FA *in vivo* (Shingfield et al., 2010b).

Biohydrogenation of long-chain unsaturated FA involves a series of bacterial metabolic reactions and the formation of multiplicity of intermediates. It has been speculated that, similarly to the already known pathways of C18 PUFA metabolism, ruminal biohydrogenation of EPA and DHA results in intermediates with 5 or 6 double bonds, containing at least one *trans* double bond (Jenkins et al., 2008). However, the first attempts to recognise it did not involve the formation of a system of conjugated bond (Kairenius et al., 2011).

LCFA in the rumen may be proceed *via* two distinct mechanisms that involve sequential reduction and/or bacterial isomerization of *cis* double bond, which is the closest to the carboxyl group (Table 1). One of the main transformations of DHA in the rumen involves the initial removal of the double bond between 4 and 5 carbon atoms followed by the reduction of the double bond at Δ7 (Kairenius et al., 2011). This is confirmed by the position of double bonds in *t-5,c-10,c-13,c-16,c-19* C22:5 identified in cow omasal digesta. Biohydrogenation of EPA involves the reduction of *cis* double bonds at Δ5, Δ8 and Δ11 (Kairenius et al., 2011).

Identification of intermediates arising in the rumen during biohydrogenation of highly unsaturated FA is of utmost importance, because it may provide an explanation of basic mechanisms involved in this process as well as better understanding of limited

Table 1. Biohydrogenation intermediates of long-chain and very long-chain unsaturated fatty acids (Kairenius et al., 2011)

Substrate		Intermediates
C20	C20:1 :	*c-11; c-13; c-14; c-15; t-9; t-10; t-11; t-12; t-13; t-14; t-15*
	C20:2 :	*c-11,t-14; c-14,c-17; c-10,t-15; t-11,c-15; t-11,t-17; t-13,t-17; t-14,t-17; t-9,t-15; t-10,t-16; t-11,t-15; t-13,t-17*
	C20:3 :	*c-8,c-11,c-14; c-11,c-14,c-17; c-11,c-14,t-17; c-10,t-14,t-17; t-10,c-14,t-17; c-11,t-14,t-17; t-11,c-14,t-17; t-11,c-14,c-17; t-10,t-14,c-17; t-9,t-14,t-17; t-10,t-14,t-17; c-10,t-14,t-18; t-10,c-14,t-18*
	C20:4 :	*c-5,c-8,c-11,c-14; c-8,c-11,c-14,c-17; t-7,c-11,c-14,c-17*
	C20:5 :	*c-5,c-8,c-11,c-14,c-17*
C21	C21:3 :	*c-12,c-15,c-18*
	C21:4 :	*c-9,c-12,c-15,c-18*
	C21:5 :	*c-6,c-9,c-12,c-15,c-18*
C22	C22:1 :	*c-11;c-13; c-15*
	C22:2 :	*t-12,t-17*
	C22:3 :	*c-10,c-13,c-16; c-10,t-14,c-19; t-12,c-16,c-19; c-10,t-13,t-17; t-10,c-13,t-17*
	C22:4 :	*c-10,c-13,c-16,c-19; c-7,t-13,c-16,c-19; t-8,c-13,c-16,c-19; t-10,t-13,c-16,c-19*
	C22:5 :	*c-7,c-10,c-13,c-16,c-19; t-5,c-10,c-13,c-16,c-19*
	C22:6 :	*c-4,c-7,c-10,c-13,c-16,c-19*
C24	C24:5 :	*c-9,c-12,c-15,c-18,c-21*
	C24:6 :	*c-6,c-9,c-12,c-15,c-18,c-21*

adipogenesis and lipogenesis occurring in ruminants fed diets supplemented with fish oil (Kairenius et al., 2011).

Presence of some other long-chain intermediates of FA was confirmed in hepatic and epididymal adipose tissues (Destaillats et al., 2005a) as well as in plasma (Plourde et al., 2006) of rats fed equimolar quantities of rumelenic and isorumelenic acids. It is claimed that RLnA was more effectively metabolized than *c-9,t-13,c-15* C18:3 isomer, because of the higher number of metabolites: six conjugated FA ranging from C16 to C22 arise from rumelenic (*c-7,t-9,c-13* C16:3; *c-11,t-13,c-17* C20:3, *c-8,c-11, t-13,c-17* C20:4, *c-5,c-8,c-11,t-13,c-17* C20:5, *c-7, c-10,c-13,t-15,c-19* C22:5, *c-4,c-7,c-10,c-13,t-15, c-19* C22:6) while only two (*c-7,t-11,c-13* C16:3 and *c-5,c-8,c-11,t-15,c-17* C20:5) from isorumelenic acid which is found to be accumulated in rat liver and epididymal adipose tissue (Destaillats et al., 2005a).

The verification how the various CLnA isomers are metabolized in the organisms of both mono- and polygastric animals and whether their chemical structures (conjugated triene or conjugated diene) influence this process seem to be very interesting cognitive aspects of future research.

Physiological activity of CLnA isomers

Physiological activity of CLnA isomers was widely studied both in humans (Yuan et al., 2009a,c) and animals (Tsuzuki et al., 2004, 2006; Yuan et al., 2009b; Nekooeian et al., 2014). Experiments with FA derived only from plant sources (especially pomegranate and bitter melon seed oils) were conducted by Ray et al. (2010) and Vroegrijk et al. (2011). It is easier to obtain an effective dose of CFA from plant sources due to the fact that concentration of CLnA isomers in these seed oils are many times higher than, e.g., in milk or meat (Modaresi et al., 2011; Razzaghi et al., 2015). To our best knowledge, the first attempts to evaluate absorption and metabolism of animal-derived CLnA isomers *in vivo* were researches of Destaillats et al. (2005a) and Plourde et al. (2006).

There are very promising results, especially regarding the antidiabetic (Nekooeian et al., 2014), anticancerogenic (Shinohara et al., 2012) and anti-obesity (Vroegrijk et al., 2011) properties of CLnA isomers. Conjugated isomers of C18:3 are also claimed to have an influence on fatty acids composition of blood (Bialek et al., 2014b) as well as metabolism of PUFA *via* lipoxygenase (LOX) pathways (Białek et al., 2016).

It is supposed, that CLnA isomers could exert beneficial properties through their metabolites or their own effect (de Carvalho et al., 2010). Some of CLnA isomers, e.g., punicic acid (*c-9,t-11, c-13* C18:3) can be converted to *c-9,t-11* C18:2 *via* Δ13-saturation reaction. This reaction is carried out by a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme that is either a novel enzyme recognizing fatty acids with three conjugated double bonds or the enzyme active in the reductive pathway of leukotriene B4 (LTB4). Therefore, sources of these CLnA are sometimes called an indirect source of CLA (Melo et al., 2016).

Diabetes

It was confirmed that pomegranate seed oil (PSO) improved insulin secretion without changing fasting blood glucose. Also reduction of the oxidative stress induced by diabetes, characterized by increased level of serum glutathione peroxidase, was observed after 28-day administration of 200 or 600 mg · kg^{-1} · d^{-1} (Nekooeian et al., 2014). The results obtained by Saha and Gosh (2009) indicate also the effectiveness of PA against sodium arsenite-induced oxidative stress.

Obesity

CLnA isomers can also altered the body composition, especially excessive weight gain (Hennessy et al., 2011). Dietary supplementation with PA caused the reduction of leptin production and increased carnitine palmitoyltransferase activity which may be one of probable mechanisms of the CLnA anti-adipogenic action (Koba et al., 2007b). This was confirmed in 12-week mice study, in which animals received high fat diet where 1 g of palm oil was replaced with PSO. PA, as the most abundant FA in PSO, prevents diet-induced obesity and insulin resistance (Vroegrijk et al., 2011).

Lipid metabolism

Prevalence of obesity is often associated with dyslipidaemia, which is a main factor of comorbidities (Yuan et al., 2014). CLnA isomers can exert hypocholesterolaemic activity strongly associated with improved cardiovascular health (Hennessy et al., 2011). The total cholesterol level in rats fed intragastrically 0.15 ml of PSO per day for 21 weeks was lower in comparison with the control group. This may prove that PSO prevents the age-related increase of cholesterol level. Unfortunately, the high density lipoprotein (HDL) fraction of cholesterol was negatively influenced by PSO supplementation.

An effective way to increase HDL concentration in blood can be joint consumption of extract of dried bitter melon fruits which is a good source of other CLnA isomer – *c-9,t-11,t-13* C18:3 (α-eleostearic acid). Incorporation of PSO into rat diet also strongly decreased the triglycerides concentration in blood (Białek et al., 2014b). Some authors also suggest that distribution of CLnA at the specific position of triacylglycerols (TAG) molecule plays an important role in reducing the adipose tissue. Punicic acid, located exclusively at the *sn-2* position was more effective in lowering fat mass than when it was distributed to all positions of TAG (Koba et al., 2007a).

Another possible mechanism by which CLnA may affect the lipid metabolism is competition with PUFA and influence the eicosanoid biosynthesis (Białek et al., 2016), especially hydroxyeicosapentaenoic (HEPE), hydroxyeicosatetraenoic (HETE), hydroxyoctadecadienoic (HODE) acids, which play a relevant role in the cancerogenesis (Jelińska et al., 2014). Production of 15-HETE, which is arachidonic acid LOX metabolite, was inhibited in serum of rats fed diet enriched with PSO in the amount of 0.15 ml per day. PA was converted to *c-9,t-11* C18:2 and both of these conjugated isomers may inhibit the activity of enzyme converting arachidonic acid to 15-HETE (Białek et al., 2016).

Anticancerogenic properties

Various CLnA isomers reveal substantially different antitumorigenic properties (Hennessy et al., 2011). It was confirmed that *all-trans* CLnA isomers: β-eleostearic (*t-9,t-11,t-13* C18:3) and β-calendic (*t-8,t-10,t-12* C18:3) acids stronger inhibit human colon cancer cell (Caco-2) growth than *partial-trans* isomers (Yasui et al., 2006). Results obtained by Shinohara et al. (2012) allowed to arrange CLnA isomers of plant origin according to their anticancer activity (Table 2). All CLnA isomers induced the apoptosis of colorectal cancer cell-lines but jacaric acid (*c-8,t-10,c-12* C18:3) made it in the greatest extent. The strongest cytotoxic effect on the prostate cancer cells was showed by jacaric and punicic acids (Gasmi and Sanderson, 2013). Cytotoxic effect of CLnA isomers of conjugated triene type was more evident than this of conjugated diene type, which is partially conjugated (Koba et al., 2007a). It is said that the mechanism of cytotoxic effect of CLnA is connected with the lipid peroxidation, which causes an activation of protein kinase C (PKC) responsible for apoptosis induction and proliferation inhibition (Grossmann et al., 2010). Nevertheless, the addition of antioxidant compounds (e.g., α-tocopherol) decreased these beneficial properties of CLnA isomers (Shinohara et al., 2012).

Taking into account all above-mentioned health-promoting characteristics of CLnA isomers, it seems to be justified to increase the content of these conjugated compounds and their metabolites in food products. Searching for strategies to increase the CLnA isomer content in animal-derived food products and yet not exhibiting negative effects on farm animal well-being and physiology is a great challenge.

Strategies to increase CLnA isomers contents in milk and meat

Effective dose of CLnA isomers for humans is 2–3 g per day (Shinohara et al., 2012). Unfortunately, it was calculated on the basis of results obtained during animal experiments, and as human metabolism and ability to absorb nutrients is different than in animals, this dose cannot be directly considered as effective and safe for humans (Fontes, 2015).

The simplest way to provide sufficient amounts of CLnA to obtain biological activity is the incorporation of plant seed oils into animal diets. Unfortunately, these oils are not commonly available worldwide due to their exotic origin, so searching for other dietary sources of CLnA isomers appears relevant. Obtaining adequate amounts of these FA from animal-derived products could be difficult because of their lower concentrations of CLnA. In regard to the fact that ruminant-derived foods significantly contribute to the total fat consumption (Wąsowska et al., 2006), efforts aimed to increase CLnA isomers content in milk and meat are of great importance.

Recently, altering the FA composition of ruminant products involved lowering the saturated fatty acids (SFA) and increasing *cis* monounsaturated (MUFA) and PUFA (especially n-3 PUFA) contents to improve the quality of fat in human diet without the need to change consumer eating habits (Shingfield et al., 2013). Composition of animal diet is considered as the main factor affecting the fatty acids profile of ruminant products (Buccioni et al., 2012) with special emphasis on type and amount of fat in the ration. So, these factors should be particularly taken into account during production of high quality milk and meat of quality adapted to nutritional requirements and consumer demands (Manso et al., 2016).

The extent to which dietary fat is incorporated into milk and muscles depends on two factors:

Table 2. Physiological effects of conjugated linolenic acid (CLnA) isomers on different health status

Health status	Model	CLnA isomer	Dosage and duration	Observed effects	References
Health	Animal (rat)	α-eleostearic acid	1 g of oil, 24 h	CLnA metabolized into c-9,t-11 CLA in liver, kidneys and small intestine mucosa	Tsuzuki et al., 2004
Health	Animal (rat)	punicic acid	0.15 ml of oil per day, 21 weeks	increased fasting glucose level; decreased total and high density lipoprotein (HDL) cholesterol content	Bialek et al., 2014b
Health	Animal (rat)	punicic acid	0.15 ml of oil per day, 21 weeks	decreased level of 15-hydroxyeicosapentaenoic acids (HEPE) and 15- hydroxyeicosatetraenoic acids (HETE); increased level of 12-HEPE and 12-HETE	Białek et al., 2016
Health	Human	punicic acid	3 g of punicic acid, 28 days	increased level of punicic acid in plasma and in red blood cell membranes (RBCM); increased proportion of *c-9,t-11* CLA in plasma and RBCM	Yuan et al., 2009c
Health	Human	punicic acid	3 g of punicic acid, 28 days	increased urinary 8-*iso*-prostaglandin F2α concentration; no effects on body weight, plasma lipid profile, cholesterol-reactive protein, interleukin 6, insulin and glucose plasma concentration and insulin resistance	Yuan et al., 2009b
Oxidative stress	Animal (rat)	α-eleostearic acid, punicic acid	0.5% of total lipids, 15 days	increased superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities; normalized cholesterol level in plasma and tissues; increased phospholipid content in liver	Saha and Ghosh, 2009
Obesity and diabetes	Animal (mouse)	punicic acid	0.25%, 4 weeks	lowered perirenal and epididymal adipose tissue weights; lowered hepatic triglyceride concentration	Koba et al., 2007b
Obesity	Animal (rat)	α-eleostearic acid, punicic acid, catalpic acid, calendic acid	0.7%, 4 weeks	decreased perirenal adipose tissue weight	Koba et al., 2007a
Obesity and insulin resistance	Animal (mouse)	punicic acid	1% of oil, 12 weeks	lowered weight gain; lowered fat mass; decreased insulin concentration; increased peripheral insulin sensitivity	Vroegrijk et al., 2011
Diabetes	Animal (rat)	punicic acid	200 mg of oil per kg per day, 600 mg of oil per kg per day, 28 days	higher levels of serum insulin and GPx activity	Nekooeian et al., 2014
Cancer	Prostate cancer cells (LNCaP, PC3 and RWPE-1)	jacaric acid, punicic acid, α-calendic acid, β-calendic acid, catalpic acid	0, 1, 3, 10, 30, 100 μM, 24 h	decreased viability of prostate cancer cells; inducted time- and concentration-dependent apoptosis and necrosis of cancer cells; activated intrinsic apoptosis of cancer cells	Gasmi and Sanderson, 2013
Cancer	Breast cancer cells (MDA-MB-231, MDA-ER α7)	punicic acid	40 μM, 48 h	affected growth of both breast cancer cell lines; inhibited cell proliferation of both cell lines; inducted apoptosis for both cell lines	Grossmann et al., 2010
Cancer	Breast cancer cells (MDA-MB-231, MDA-ER α7)	α-eleostearic	1, 2, 5% of bitter melon extract, 6 and 24 h	inhibited breast cancer cell proliferation; induced apoptotic cell death; enhanced p53, p21 and pChk1/2 and inhibited cyclin B1 and cyclin D1 expressions; modulated cell cycle	Ray et al., 2010
Cancer	Colorectal adenocarcinoma tumor cells (DLD-1)	punicic acid, catalpic acid, α-eleostearic acid, β-eleostearic acid, jacaric acid, α-calendic acid, β-calendic acid	0, 5, 10 μmol · l^{-1}, 6, 12, 24 h	decreased survival of DLD-1 cells; induced apoptosis of DLD-1 cells; increased level of lipid hydroperoxide (LOOH) in cells	Shinohara et al., 2012
Cancer	Animal (mouse) with transplantated DLD-1 cell	jacaric acid	1 mg of oil per day per mouse, 36 days	reduced tumor size; higher lipid peroxidation level of tumor tissues; higher percentage of triphosphate nick-end labeling (TUNEL)-positive cells in tumor tissues	Shinohara et al., 2012

biohydrogenation of UFA in the rumen and the efficiency of FA transfer from the small intestine. Transfer of C18 fatty acids into milk is regulated by the bioaccumulation of fatty acids from diet (Glasser et al., 2008). The potential to affect the fatty acid composition of muscles is mainly determined by the lipolysis and biohydrogenation of dietary lipids in the rumen. In contrast to milk, it is possible to influence the content of C20 PUFA in tissues by supplementing the diet with sources of linoleic and α-linolenic fatty acids, as well as dietary supplements containing 20-carbon PUFA (Sinclair, 2007; Doreau et al., 2011). So, the manipulation in ruminant diet seems to be the most effective way to introduce CLnA isomers into their milk and meat (Fontes, 2015).

There are some studies indicating that incorporation of linseed (Mapiye et al., 2013a,b; Ebrahimi et al., 2014) and linseed oils (Bessa et al., 2007) into cattle and lamb diets may result in accretion of CLnA isomers in their tissues. Supplementation of lucerne basal diet with 7.4% of linseed oil lead to the occurrence of rumelenic acid in the *longissimus thoracis* muscle at the level of 329 mg · 100 g^{-1} FA (Bessa et al., 2007). Linseed increased rumelenic acid content in intramuscular fat of steers fed both red clover silage (Mapiye et al., 2013a) and high-forage (Mapiye et al., 2013b) diets by 0.15 mg · g^{-1} tissue and 0.13 mg · g^{-1} tissue, respectively. Also, rumelenic acid was detected in the *semitendinosus* muscle of kid goat fed diet containing 1.30% linseed (0.41% FA) (Ebrahimi et al., 2014). In milk of cows fed diet with raw and extruded linseed CLnA constituted 0.15 and 0.18% of fatty acids, respectively in comparison to the control group, where CLnA content was under the rejection threshold (Akraim et al., 2007).

As evidenced above, the addition of ALA sources to the animal rations result only in minor increase of CLnA isomers content in their milk and meat. Several authors had also investigated an effect of pomegranate by-products (i.e. seed pulp) remaining after juicing of pomegranate fruits (Modaresi et al., 2011; Kotsampasi et al., 2014; Razzaghi et al., 2015) on fatty acid composition of goat and lamb products. As claimed by Modaresi et al. (2011) 12% pomegranate seed pulp can effectively increase both PA and ALA contents in goat milk fat, since PA constitutes about 75% of total FA in dietary pulp. Similar results were obtained when pomegranate seed pulp was administrated to animals in the amount of 120 g · kg^{-1} dry matter (Razzaghi et al., 2015). On the other hand, ensiled pomegranate by-products used as lamb diets supplement by Kotsampasi et al. (2014) did not contain even trace amounts of CLnA isomers, which was reflected in the milk fatty acid profile. This may be caused by the fact that the amount of seeds (where the PA is most abundant) was insufficiently accounted for the whole pulp. Considering also different chemical composition of individual parts of fruits like peel, seeds or arils (Chaturvedula and Indra, 2011), it can be assumed that demonstrated effects are the result of a combination of multiple interactions between bioactive components of the fruit. So, the assumption, that each fruit component used in animal nutrition separately may exert different features, seems to be justified. Therefore in order to recognize the precise mechanism of physiological changes in animal organism, it is important to eliminate both antagonistic and synergistic effects. The investigation of 'pure' sources of CLnA (such as plant oils) metabolized/biohydrogenated in ruminants is very promising perspective for future research.

Analytical aspects of CLnA isomers

The proper identification of biohydrogenation intermediates of UFA is a great analytical challenge, mainly due to the relative small concentrations of these compounds in assayed samples, lack of their standards, frequent co-elution as well as poor resolution (Alves and Bessa, 2014). Whereas the most important task in analysis of conjugated fatty acids (CFA) is to establish the position of double bond and its geometrical configuration (*cis*/*trans*) in the carbon chain of FA.

The location of double bond in the chain of UFA was frequently investigated using the gas chromatography coupled with mass spectrometry (GC-MS) analysis of 4,4-dimethyloxazoline (DMOX) derivatives of FA (Wąsowska et al., 2006; Halmemies-Beauchet-Filleau et al., 2011; Lerch et al., 2012). Because the carboxyl group (-C(=O)OH) is known to be highly sensitive to fragmentation and double bond migration, the stabilization of this group by the formation of nitrogen containing derivative (i.e. DMOX) allows for the structural determination of most FA (Plourde et al., 2007). Spectra of SFA DMOX derivatives are recognized by regular 14 atomic mass units (amu) gaps between adjacent methylene groups (CH3), while in unsaturated chain – this is the gap of 12 amu. It is interpreted as cleavage of the double bond in fatty acid chain (Wąsowska et al., 2006). When CFA are considered, some characteristic type of fragmentation, and thus the most intense ion, is associated with bis-methyl-

ene-interrupted dienes as well as with tris-methylene-interrupted dienes, and can serve as a diagnostic fragment to locate double bonds in these fatty acids (Alves and Bessa, 2014), e.g., intense ion fragment at m/z 264 is an indicative feature of bis-methylene-interrupted diene with double bonds in C11 and C15 positions (Wąsowska et al., 2006) while abundant ions at m/z 182, 288 and 302 supports the occurrence of double bonds at C9, C11 and C13, respectively (Lerch et al., 2012). GC-MS of DMOX derivatives of FA would require the combined use of other techniques for CFA isomers identification to determine *cis* or *trans* configuration (de la Fuente et al., 2006).

The covalent adduct chemical ionization (CACI) coupled with tandem mass spectrometry (MS/MS) also allows to identify the double bond position, but without the need of prior derivatization (Alves and Bessa, 2014). Under CACI condition, a reagent (e.g., acetonitrile) is subjected to the chemical ionization (CI). Then an ionized reagent reacts with itself to produce a reagent ion; next this ion reacts with analyte double bond with a set of different ions arising. These collision-activated ions in each analyte yield in specific diagnostic fragments, which are associated with the presence of double bond in particular position of chain (Alves and Bessa, 2014).

Summing up, GC-MS of DMOX derivatives provide information about the position of double bond whereas CACI-MS gives only some evidence for its geometrical configuration (Gómez-Cortés et al., 2009). Unfortunately, these techniques are not capable to the unequivocal assignment of geometric configuration of double bond. It can be only deduced on the basis of the elution order (Gómez-Cortés et al., 2009) and relative retention times (Honkanen et al., 2016) of known isomers. Due to the fact, that during biohydrogenation may arise multitude of intermediates, also conjugated ones, and some of them may be unidentified yet, the simple, specific and efficient method with satisfactory accuracy and sensitivity is required for their exact geometric configuration identification.

For many years, silver-ion/argentation liquid chromatography has become an important method for fractionation of fatty acids, mainly due to its ability to separate them accordingly to the configuration as well as the number and position of their double bonds (de la Fuente et al., 2006). Silver ion liquid chromatography (Ag^+-HPLC) coupled with a photodiode array detector (DAD) is suggested for direct determination of underivatized and methylated CLA isomers in various biological samples (Roach et al., 2002; Czauderna et al., 2003, 2011). According to AOAC (2005) this method was described as suitable also for determination of other conjugated fatty acids (trienes, tetraenes, pentaenes) (Fritsche et al., 2000), while Ag^+-TLC (silver-ion thin layer chromatography) is claimed not to resolve di-, tri-, tetra-, penta- and hexaenoic fatty acids (Kairenius et al., 2011). Pi (π) electrons of fatty acid double bonds react reversibly with silver ions to form polar complexes, while residual silanol groups interact with carboxylic acid groups of FA (Czauderna et al., 2003). Thus the balance of forces contributing to retention is altered (Cross and Zackari, 2002) – retention increases along with the increasing number of double bonds in the chain, as well as with *cis* over *trans* configuration (Turner et al., 2015). Silver ions interact with π electrons of *cis* double bonds better than with *trans* ones, which results in faster elution of *trans*-isomers (Stolyhwo and Rutkowska, 2013). High-resolution Ag^+-HPLC is better method to separate positional CFA isomers among all geometrical groups than reversed-phase HPLC (RP-HPLC) and capillary gas chromatography techniques (Czauderna et al., 2011). Preponderance of Ag^+-HPLC method also over gas chromatography had been proved during analysis of *trans*-isomers profile of milk fat. GC with a flame ionization detector (FID) analysis enabled only partial separation of *trans*-isomers from other FA in sample, while Ag^+-HPLC allows to separate particular FA classes according to their unsaturation degree and geometric configuration (Stolyhwo and Rutkowska, 2013). So, the high-resolution Ag^+-HPLC with DAD seems to be the most appropriate analytical technique for precise, accurate, specific and sensitive fractionation, discrimination and quantification of various CFA isomers in samples of various origin.

Conclusions

The presence of conjugated linolenic acid (CLnA) isomers in human foods is mostly associated with bacterial isomerization of α-linolenic acid in the rumen of farm animals as well as in some plant products (like pomegranate or bitter melon seed oils). The majority of latest clinical studies documented that numerous physiological properties have been attributed to CLnA isomers as anti-adipogenic, antidiabetogenic, antiatherosclerotic and anticarcinogenic agents. Our review of the world's scientific literature provide a valuable insight into the physiological actions of CLnA isomers in mammals. It is especially important to note that the

long-term effects of CLnA isomers are still unknown, therefore they should be addressed before recommending CLnA isomer supplementation to humans. Moreover, optimal CLnA isomer, timing, and dosage along with the effects of energy intake and body mass index in humans remain unclear. Lack of such knowledge impedes the development of isomer-specific, CLnA isomer fortified foods or supplements for the prevention or treatment of obesity and cardiovascular diseases, the most prevalent nutrition-related diseases in western countries (especially in USA and UK). Our review suggests a need of future clinical studies focusing on a desirable insight into the physiological actions of individual CLnA isomers in humans.

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