Evidences and perspectives in the utilization of CLNA isomers as bioactive compounds in foods

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ABSTRACT
Conjugated alpha linolenic acid (CLNA) isomers are promising lipids owing to their similarities with conjugated linoleic acid (CLA) but exerting their bioactivity at lower doses; some isomers also belong to omega 3 family. This review aims to summarize the state of the art about the utilization of CLNA as a functional ingredient. Indeed, in vitro and in vivo studies reported that CLNA exerted anticancer, anti-inflammatory, anti-obese, and antioxidant activities. However, CLNA has not been tested in humans. These compounds are naturally present in meat and milk fat from ruminants but the highest concentrations are found in vegetable oils. Their incorporation in foodstuffs is one of the most effective strategies to elaborate CLNA-enriched products together with the microbiological production. Lactobacilli, propionibacteria, and bifidobacteria strains have been assayed to produce CLNA isomers but at the current moment there are not high CLNA concentration products elaborated using these strains. Furthermore, it is known that CLNA isomers are highly prone to oxidation when compared with linoleic acid and CLA, but the possible effects of elaboration and storage on high CLNA products are unknown. The utilization of CLNA as a functional compound still remains a challenge and requires more research to address all of its technological and bioactivity aspects.

KEYWORDS
CLNA-enriched products; microbiological production; bioactivity; stability; safety

Introduction
Cardiovascular diseases (CVD), such as ischemic heart disease and stroke, have been the leading cause of deaths worldwide, being responsible for 17 million deaths in 2008 (Alwan, 2011). Overweight and obesity are the major risk factors for CVD, and it was estimated that between 1980 and 2008 the prevalence of overweight and obese adults (≥20 years old) increased from 24.6% to 34.4% and from 6.4% to 12.0%, respectively (Stevens et al., 2012). Although lipids are involved in the development of these conditions, recent studies have also stated their positive health effects on humans. Thus, dietary sources of omega 3 have shown to be capable of reducing the incidence of CVD through decreasing the number of sudden deaths as well as the effect of risk factors such as obesity, hypertension, and cholesterol (Harris, 2008). Furthermore, the bioactivity of conjugated linolenic acid (CLNA) has been well characterized, namely for its anticarcinogenic, anti-obese, anti-diabetic, and anti-hypertensive effects (Koba and Yanagita, 2014). However, CLA is not the only bioactive conjugated fatty acid, as recently conjugated alpha linolenic acid (CLNA) has also shown bioactive potential as anti-carcinogenic, anti-inflammatory, anti-obese, and antioxidant compounds (Yuan et al., 2014). Thus, this review aims to summarize the evidences about the beneficial health effects of CLNA and the possibilities of their utilization in new functional foodstuffs.

CLNA in foods: Main sources and contents
It has been reported that CLNA isomers occur naturally in milk fat and meat of ruminants; however, it is mostly found in vegetable oils (Mapiye et al., 2013) (Table 1). In spite of all the possible isomers, only seven compounds are found in plant seed oils: jacaric acid (JA) (C18:3 c8,t10,c12), α-eleostearic acid (α-ESA) (C18:3 c9,t11,t13), β-eleostearic acid (β-ESA) (C18:3 t9,t11,t13), punnicic acid (PUA) (C18:3 c9,t11,c13), α-calendic acid (α-CDA) (C18:3 t8,t10,c12), β-calendic acid (β-CDA) (C18:3 t8,t10,t12), and catalpic acid (CPA) (C18:3 t9,t11,c13) (Tanaka et al., 2011). The main source of JA is the seed oil of argentine native tree Jacaranda mimosifolia, with 36 g/100 g of oil (Kraus et al., 2005). The isomer α-ESA is the main compound of tung oil (Aleurites fordii) (>70 g/100 g of oil), a native tree from Southeast Asia and the Pacific Islands (Burrows and Tyrl, 2013), bitter melon (Momordica charantia) (>50 g/100 g of oil) (Dhar et al., 1999), and Parinarium spp. (>60 g/100 g of oil) (Scrimgeour and Harwood, 2007). This isomer can be found in white mahlab (Prunus mahaleb) (~40 g/100 g of oil), a small tree native of southern Europe that grows wild in the Mediterranean region across Turkey (Sbihi et al., 2014).
Tung and bitter melon seeds also contain β-ESA but in lower concentrations (3.5 and 2.6 mol/100 mol of oil, respectively) than α-ESA (Tsuzuki et al., 2004). PUA is mainly found in pomegranate (*Punica granatum*) (>70 g/100 g of oil) (Spilmont et al., 2013) and balsam apple (*Momordica balsamina*) (~50 g/100 g of oil) (Gaydou et al., 1987), while snake gourd of *Trichosanthes anguina* and *Trichosanthes kirilowii* species have approximately 40 g/100 g of oil and above 30 g/100 g of oil, respectively (Yang et al., 2012). *Trichosanthes anguina* is a native vine from tropical Asia and is cultivated in China, while *Trichosanthes kirilowii* is distributed through several Chinese provinces and also occurs in South Korea and Japan (Hu, 2005). In pot marigold (*Calendula officinalis*), it is possible to find α-CDA (>50 g/100 g of oil) and a small amount of β-CDA (<1 g/100 g of oil) (Dulf et al., 2013). CPA is mainly present in *Catalpa ovata* (>40 g/100 g of oil), a species originating in China (Suzuki et al., 2006). Among the seeds that contain CLNA isomers, only those from pomegranate, snake gourd *T. kirilowii*, and white mahaleb are edible. Pomegranate is native of Persia and cultivated in Mediterranean countries, Afghanistan, Iran, China, Japan, Russia, and some parts of the United States. Its edible parts comprise 80% juice and 20% seeds, and are used to produce fresh juice, syrups, canned beverages, jelly, jam, and flavoring drinks (Fadavi et al., 2006). *T. kirilowii* seeds have long been used as a traditional Chinese medicine to reduce infections and are a popular snack food from China (Yuan et al., 2014). White mahaleb powder is used in Greece, Cyprus, Turkey, and neighboring Arab countries of Syria and Saudi Arabia for flavoring breads and pastries, while in Sudan it is also used as a medicine for children in diarrheoider et al., 2012).

| Isomers       | Chemical structure | Source                                | Amount                       | References                       |
|---------------|--------------------|---------------------------------------|------------------------------|----------------------------------|
| Jacaric acid (JA) | C18:3 c8t10c12   | Jacaranda mimosifolia seed oil        | 36 g/100 g of oil            | Tulloch, 1982                    |
| α-elaostearic acid (α-ESA) | C18:3 c9t11t13   | Aleurites fordii seed oil             | >70 g/100 g of oil           | Tsuzuki et al., 2006             |
| Momordica charantia seed oil |               |                                      | >50 g/100 g of oil           | Dhar et al., 1999                |
| Parinam spp. seed oil |               |                                      | >60 g/100 g of oil           | Scrimgeour and Harwood, 2007     |
| Rumelenic acid (RLA) | C18:3 t9t11t13   | Aleurites fordii seed oil             | 3.5 mol/100 mol of oil       | Tsuzuki et al., 2004             |
| β-elaostearic acid (β-ESA) | C18:3 t9t11t13   | Aleurites fordii seed oil             | 2.6 mol/100 mol of oil       | Tsuzuki et al., 2004             |
| Punicic acid (PUA) | C18:3 c9t11c13   | Punicia granatum seed oil             | >70 g/100 g of oil           | Splimont et al., 2013            |
| Momordica balsamina seed oil |               |                                      | ~50 g/100 g of oil           | Gaydou et al., 1987              |
| Trichosanthes anguina seed oil |               |                                      | ~40 g/100 g of oil           | Mukherjee et al., 2002           |
| Trichosanthes kirilowii seed oil |               |                                      | ~30 g/100 g of oil           | Yang et al., 2012                |
|α-calendic acid (α-CDA) | C18:3 t8t10c12   | Calendula officinalis seed oil        | <1 g/100 g of oil            | Dulf et al., 2013                |
| β-calendic acid (β-CDA) | C18:3 t8t10c12   | Calendula officinalis seed oil        | >40 g/100 g of oil           | Suzuki et al., 2006              |
| Catalic acid (CPA) | C18:3 t9t11c13   | Catalpa ovata seed oil                | 0.03–0.39 g/100 g of fat     | Plourde et al., 2007a; Lerch et al., 2012; |
| Rumelenic acid (RLA) | C18:3 c9t11c15   | Bovine milk                           | 0.06–0.08 mg/g of muscle     | Nassu et al., 2011; Mapiye et al., 2013 |
| Rumelenic acid (RLA) | C18:3 c9t11c15   | Bovine meat                           | 0.29 g/100 g of meat fat     | Ebrahimi et al., 2014            |
| Goat meat      |                   |                                      | 0.02–0.06 mg/100 g of fat    | Lerch et al., 2012               |
| Goat meat      |                   |                                      | 0.02 mg/g of muscle         | Nassu et al., 2011; Mapiye et al., 2013 |
| rumi         |                   |                                      | 0.03 g/100 g of meat fat     | Ebrahimi et al., 2014            |

In comparison to vegetable oils, the concentration of CLNA in these foodstuffs is very low, which suggests that the intake of CLNA, in order to highlight its potential health benefits, would not be viable through this natural source, therefore, strategies to enhance its intake are to be studied.

**CLNA bioactivity**

**Anticancer activity**

Some studies have reported the cytotoxic effect of CLNA isomers on different human tumor cell lines, including MDA-MB-231, MCF-7 (breast) (Moon et al., 2010), HT-29 (colon) (Degen et al., 2011), MDA-MB-231 (estrogen insensitive breast cancer cells), MDA-ERα7 (estrogen sensitive breast cancer cells cloned from MDA-MB-231 cells) (Grossmann et al., 2010), T24 (bladder) (Sun et al., 2012), HeLa (cervix) (Eom et al., 2010), LNCaP, PC-3 (prostate) (Gasmiri and Sanderson, 2013), and DLD-1 (colorectal) (Shinohara et al., 2012b). These works found that the anti-proliferative activity was due to an increment in the number of cells in G0/G1 phase (cells are arrested in the initial phases of cell cycle and do not continue to proliferate), and the increase of apoptosis rate is the main anticancer activity evidenced for CLNA isomers.

These activities seem to be influenced by the configuration of double bonds. Degen et al. (2011), when evaluating growth inhibition effects of pure CLNA isomers against HT-29 colon cancer cells, found that in the presence of an all-trans isomer, such as C18:3 t9t11t13, it resulted in greater inhibition than with C18:3 c9t11t13. Furthermore, Shinohara et al. (2012b), assaying the cytotoxic effects of several pure CLNA isomers from vegetable sources on DLD-1 colorectal cancer cells, observed that JA (C18:3 c8t10c12) exerted a stronger effect in terms of decreasing cell survival and inducing apoptosis when compared with α-ESA (C18:3 c9t11t13), PUA (C18:3 c9t11t13), CPA (C18:3 t9t11t13), and the trans-isomers β-ESA (C18:3 t9t11t13) and α-CDA (C18:3 t8t10t12), all tested at the same concentration (10 μM). Further studies partially confirmed these previous
results since when pure JA, PUA, α-CDA, and β-CDA cytotoxicity was investigated against LNCaP and PC-3 prostate cancer cells, results pointed out that PUA and JA as the most effective isomers (Gasmì and Sanderson, 2013). At the time PUA and JA 3-D conformations were analyzed and overlapped, shape and feature similarity values were highly correlated, indicating that the cis, trans, cyc configurations of their double bonds are the reasons of their bioactivity.

It has been also suggested that the mechanism of CLNA anticancer activity may be related to lipid peroxidation, since the addition of α-tocotrienol, an antioxidant, leads to the loss of PUA cytotoxic properties against MDA-MB-231 and MDA-ERα7 breast cancer cells (Grossmann et al., 2010). These authors also associated the CLNA’s cytotoxicity to the induction of protein kinase C (PKC) that leads to the inhibition of cell proliferation and activation of apoptosis. Other studies have reported increase of caspase-3 expression, decrease of apoptosis suppression factor Bcl-2 expression, formation of reactive oxygen species (ROS), activation of caspase-9 cascade, DNA fragmentation, poly ADP-ribose polymerase (PARP) cleavage, increase of tumor suppressor gene p53 expression, activation of peroxisome proliferator-activated receptor gamma (PPARγ), and inhibition of extracellular signal-regulated kinases 1 and 2 (ERK 1/2) (Moon et al., 2010; Sun et al., 2012).

Conjugated alpha linolenic acid’s anti-carcinogenic activity has been also assayed in vivo, mainly with natural plant sources. Tung oil, which is rich in α-ESA, when administered at doses of 50 and 100 mg/kg body weight to mice transplanted with DLD-1 colorectal cancer cells, suppressed vessel formation: in doses of 100 mg/kg body weight tumor vessel length was significantly lower than in 50 mg/kg body weight (Tszuzuki and Kawakami, 2008). Bitter gourd seed oil (rich in α-ESA) was also able to inhibit aberrant crypt foci (ACF) formation, induced by azoxymethane (AOM) in rats fed with at 0.01, 0.1, and 1% (Kohno et al., 2002). Furthermore, feeding mice treated with nitrobenzene, a genotoxic inducer, with 100, 200, and 400 mg/kg body weight of pomegranate seed oil (PUA-rich source), caused significant reduction in the percentage of aberrant cells and sperm shape and chromosome aberrations (Aly et al., 2014). When pomegranate seed oil was administered to rats, it led to a significant suppression of adenocarcinoma incidence and multiplicity in colon. Most of the studies described above have associated CLNA cytotoxicity in vivo to enhanced PPARγ expression. CLNA anticancer properties could also be due to the metabolism of CLNA to CLA, since lipid analysis on liver and colon in colon. Most of the studies described above have associated significant suppression of adenocarcinoma incidence and multiplicity in colon. Most of the studies described above have associated

**Anti-Inflammatory activity**

Cells (e.g., neutrophils, macrophages, and monocytes) and bio-chemicals, such as tumor necrosis factor (TNF-α), interleukins (IL), platelet activating factor (PAF), leukotrienes, and ROS, are involved in inflammatory diseases. Therefore, an increment in these mediators is an indicator of occurring inflammation.

Anti-inflammatory activity of CLNA in vitro has only been reported for PUA on human breast cancer cells (MDA-MB-231 and MCF-7) in the presence of pomegranate seed oil for 24 h at 37°C (Costantini et al., 2014). The levels of nine pro-inflammatory cytokines, including ILs and TNF-α, were significantly decreased at amounts ranging from 0.24 to 0.6 μL.

Necrotizing enterocolitis (NEC) is characterized pathologically by inflammatory and coagulative necrosis that occurs throughout the intestinal tract of, especially, newborns. Pomegranate seed oil administration at 1.5% to NEC rats caused significant reduction in ileal damage, while expression levels of IL-6, IL-8, IL-12, IL-23, and TNF-α mRNA were significantly lower (Coursodon-Boydell et al., 2012).

On the other hand, inflammatory bowel disease (IBD) is a chronic inflammatory disease that can manifest itself as ulcerative colitis (affects only the large intestine), or Crohn’s disease affects the whole digestive tract. α-ESA was shown to be able to ameliorate IBD phenotypes when incorporated in-induced IBN mouse diet through equally PPARγ-dependent and independent mechanisms (Lewis et al., 2011). Both α-ESA and PUA were also capable to significantly reduce IL-6, IL-1β, and TNF-α expressions in rats treated with sodium arsenite and streptozocin (Sahin and Ghosh, 2012; Saha et al., 2012b). According to Saha and Ghosh (2011), high concentrations of arachidonic acid (ARA: C20:4, c8,c11,c13,c16) together with low levels of γ-linolenic acid (γ-LNA: C18:3 c6,c9,c12) in tissues are indicators of inflammation. Vegetable oils containing α-ESA or PUA were capable of normalizing ARA and γ-LNA amounts in rats when an inflammatory process altered their concentrations. Both in vitro and in vivo studies suggest that CLNA can interfere in inflammation mediators. At the current moment, the anti-inflammatory effects of CLNA have not been tested on humans.

**Antioxidant activity**

Some works have reported also antioxidant properties for CLNA. Karela seed oil (rich in α-ESA) was added at 0.05 and 0.1% to human blood samples of diabetic and non-diabetic subjects, reducing lipid peroxidation at both doses in samples from diabetic subjects (Dhar et al., 2007). Other assays comparing bitter (α-ESA) and snake (PUA) gourd seed oils revealed that the antioxidant activity was greater for bitter gourd seed oil, possibly due to better α-ESA oxidative stability (Saha et al., 2012c), since trans-double bonds are more stable than cis.

Some in vivo studies have been carried out to test the activity of CLNA isomers against chemically induced oxidative stress. As in the in vitro research works mentioned above, α-ESA and PUA showed antioxidant properties in rats treated with sodium arsenite to induce oxidative stress being α-ESA, which is most effective at the lowest dose as it increased the activity of antioxidant enzymes (SOD, CAT and GPx) and glutathione (GSH) levels and decreased nitric oxide synthase (NOS) activity and lipid and protein oxidation in the plasma and kidney (Saha and Ghosh, 2013). Some of these effects were also observed with
α-ESA against the oxidative stress caused by methyl mercury and induced diabetes in rats (Paul et al., 2014).

On the other hand, the effect of PUA was tested on 15 healthy young humans, consuming an equivalent of 3 g/day of PUA from Trichosanthes kirilowii seeds for 28 days (Yuan et al., 2009); results concluded that PUA exhibited a pro-oxidant activity. These findings seem to be controversial with the in vivo studies described above; however, animal studies revealed that PUA has both antioxidant and pro-oxidant activity depending on the dose used: feeding rats with an equivalent of 0.6 g/kg of PUA lead to antioxidant effects, whereas a higher dose of 1.2 g/kg caused pro-oxidant activity (Mukherjee et al., 2002).

**Anti-obesity activity**

Conjugated linolenic acid isomers have been described to exert positive effects on body weight, and due to similarities among these fatty acids, some works have been focused in the study of the possible anti-obesity properties of CLNA. When 3T3-L1 adipose cells were exposed to a mixture of two CLNA isomers, RLA and C18:3 c9,t13,c15 at 10 and 100μM, triacylglycerol content decreased at both concentrations (Miranda et al., 2011). However, a dose of 10 μM increased the expression hormone-sensitive lipase (HSL), while 100 μM affected adipose triglyceride lipase (ATL).

Moreover, oral administration of α-ESA and PUA caused decrease of triacylglycerol (TAG), cholesterol, and LDL-C levels and increase of HDL-C level in the plasma, erythrocytes, liver, and brain of obese and hypercholesterolemic rats (Saha et al., 2012a; Sengupta et al., 2015). Although no significant weight loss was observed, body weight gain and fat mass were reduced. Interestingly, the activity of HMG-CoA reductase, responsible for cholesterol biosynthesis, was found to decrease. The authors concluded that α-ESA was more effective than PUA and associated such effects to the trans configuration of this fatty acid.

It has been elsewhere suggested that effects of CLNA on lipolysis are due to the activation of cAMP-activated protein kinase (PKA) pathway and apoptosis in white adipose tissue (Chen et al., 2012). Nevertheless, the administration of JA (5 mg/day) for one week to normal rats increased the accumulation of palmitic acid (C16:0) and stearic acid (C18:0) while decreasing palmitoleic acid (C16:1 c9) and oleic acid (C18:1 c9) in the liver and white adipose tissues (Shinohara et al., 2012a). The results were associated to the inhibition of stearoyl coenzyme A desaturase (SCD) activity, an endoplasmic reticulum enzyme that catalyzes the biosynthesis of MUFA from SFA, since the expression level of SCD-1 mRNA was significantly decreased.

Recently, a study was carried out with humans. The effect of pomegranate seed oil was investigated by monitoring lipid profile of 23 volunteer hyperlipidaemic subjects (aged <20 years) enrolled in a parallel, randomized, doubleblind, and placebo-controlled study (Mirmiran et al., 2010). The test group consumed one capsule containing 400 mg of seed oil twice a day for fourweeks. The results revealed a significant decrease in TAG and TAG:HDL-C ratio levels, although cholesterol and LDL-C levels remained unchanged.

According to the already reported research works, there are strong evidences supporting the bioactivity of CLNA isomers (mainly α-ESA and PUA) and their potential to be used as a functional ingredient. However, little research has been performed on humans. Thus, to fully understand the mechanisms and possibilities of these compounds, more investigations focusing on this topic have to be accomplished.

**Strategies for CLNA enhancement in food**

It is accepted that the effective dose of CLA to obtain the beneficial effects in humans is 3 g/day (Ip et al., 1994) and for CLNA 2–3 g/day (Shinohara et al., 2012b). However, both values were assumed based on animal models. Specifically, the CLNA dose was calculated from the amount of jatarran seed oil administered to mice (1 mg/day), exerting anticancer properties against transplanted human colorectal adenocarcinoma cells. Since it is a value obtained from animal studies, it cannot be completely assumed as the correct effective dose for humans as our metabolism and absorption of nutrients is different from that in animals.

Furthermore, obtaining this amount from milk and meat of ruminants would be difficult according to the concentration of CLNA in these foodstuffs. On the other hand, the vegetable oils may be an alternative, but pomegranate seed oil is the only one that is currently commercialized among those edible. No side effects were reported when this vegetable oil was administered to hyperlipidaemic subjects; however, the dose administered (800 mg/day of oil that is equivalent to 560 mg/day of PUA) is much lower than the above-mentioned effective dose (Mirmiran et al., 2010). Moreover, pomegranate seed oil has been successfully incorporated in margarine (0.5 g/100 g PUA), and goat milk was naturally enriched with PUA (1.19 g/100 g of fat) using 12% of pomegranate seed pulp added to the feeding of animals (Modaresi et al., 2011; Franczyk-Zarów et al., 2014).

Some other studies have assayed a different approach: the production of enriched supplements such as nanoparticles and nano-emulsions containing vegetable oils rich in CLNA isomers (50 mg/kg body weight/day in nanoparticles, and 2 g in nano-emulsions) (Paul et al., 2014; Sengupta et al., 2015). These matrices were used to enhance CLNA bioavailability and stability, and according to the results, were efficient in attenuating the effects of hypercholesterolemia and diabetes in an animal model.

The manipulation of ruminants’ diet leads to interesting results in the enhancement of CLA concentrations in milk and ruminant meat, and this has been assayed for CLNA. The studies focused on the addition of extruded seeds or oils rich in LNA, but in general the results were very limited (Mapiye et al., 2013; Ebrahimi et al., 2014).

**Microbiological production of CLNA: From rumen to cultures**

Ruminant products, such as meat, milk, and other dairy foods, represent the main source of CLA for humans. CLA isomers are formed during LA (C18:2 c9,c12) and α-LNA (C18:3 c9,c12,c15) biohydrogenation process that occurs in rumen, a multi-step pathway carried out by different microorganisms on unsaturated fatty acids (Fig. 1). Once in the rumen, LA and α-LNA from diet are hydrolyzed through microbial lipases from Butyribrio fibrisolvens (hydrolyses
phospholipids) and Anaerovibrio lipolytica (hydrolyses di- and triacylglycerols) for further reactions of isomerization and hydrogenation (Buccioni et al., 2012). The biohydrogenation of LA involves two main steps: isomerization of LA to C18:2 c9,t11 (CLA) and hydrogenation of the cis-double bond of conjugated diene to yield C18:1 t11 (trans-vaccenic acid, TVA), which is further hydrogenated to stearic acid (C18:0), while C18:2 9,11 isomers, C18:2 8,10, and C18:2 t10,c12 isomers are also produced through this pathway (Chilliard et al., 2007). LNA biohydrogenation involves similar reactions in rumen, differing in intermediate products, yielding CLA and CLNA: after isomerization, fatty acid is first isomerized at cis-12 position, producing RLA. Subsequently, this compound is reduced to C18:1 t11,c15 and further converted to three different products: C18:1 t11 (TVA); C18:1 c15, and C18:1 t15. Only TVA is reduced to stearic acid (C18:0) (Van Nieuwenhove et al., 2012). Previous works have suggested that CLA isomers can be formed from α-LNA as well, namely, C18:2 c9,c11, C18:2 t8,t10, C18:2 c10,c12, and C18:2 11,13 (Lee and Jenkins, 2011). All intermediates of biohydrogenation are absorbed in the gut and transported through the blood stream to different body tissues, thus CLA and CLNA can appear in milk and meat fat (Gorissen et al., 2013). CLA is also produced in the mammary glands of lactating cows through the conversion of TVA coming from rumen by the Δ9-desaturase. This pathway represents the primary source of CLA in milk (64%; Griinari et al., 2000). On the other hand, CLNA does not have another synthesis pathway yet evidenced, so all ruminant milk contents apparently come exclusively from α-LNA biohydrogenation and ruminants diet.

The main ruminal bacteria that is involved in the biohydrogenation process is Butyryrivibrio fibrisolvens, whose mechanism of transformation is carried out by linoleate isomerase bound to the bacterial membrane (Bauman et al., 1999). However, it has been reported that ruminal bacteria are not the only one capable of producing CLA. Species isolated from dairy products and human intestines showed a similar capacity, namely, strains of lactobacilli, bifidobacteria, and propionibacteria. Therefore, it has been proposed that other microorganisms can

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**Figure 1.** Scheme of LA and LNA biohydrogenation. Bold arrows indicate the principal pathway, and simple arrows indicate secondary pathways.
produce CLNA, or others are able to produce CLA. This hypothesis has been confirmed in some studies using strains of lactobacilli, propionibacteria, and bifidobacteria (Table 2).

**Table 2. CLNA-producing strains, culture growth conditions (medium/LNA), temperature (T), and time (t), LNA conversion rate (%), and isomer(s) formed.**

| Strain                        | Culture medium | LNA (mg/mL) | T (°C) | t (h) | LNA conversion rate (%) | Isomer(s) produced | References                           |
|-------------------------------|----------------|-------------|--------|-------|-------------------------|--------------------|--------------------------------------|
| Lactobacillus plantarum       | MRS            | 63          | 37     | 72    | 40%                     | C18:3 9,11,t15     | Kishino et al., 2003                 |
|                               | MRS            | 4           | 37     | 48    | 47%                     | C18:3 9,11,t15     | Kishino et al., 2009                 |
| Lactobacillus sakei           | MRS            | 0.5         | 30     | 72    | 60.1%                   | C18:3 9,11,t15     | Gorissen et al., 2011               |
| Propionibacterium freudenreichii, subsp. shermanii | BHI | 20 µg/mL | 37 | 48 |                      | C18:3 9,11,t15     | Verhulst et al., 1987              |
| Propionibacterium acidipropionici | BHI           | 20 µg/mL | 37 | 48 |                          |                    |                                      |
| Propionibacterium freudenreichii, subsp. shermanii | BHI | 20 µg/mL | 37 | 48 |                          |                    |                                      |
| Propionibacterium freudenreichii, subsp. shermanii | BHI | 20 µg/mL | 37 | 48 |                          |                    |                                      |
| Bifidobacterium breve         | BHI            | 0.24         | 37     | 42    | 67.6–80.7%              | C18:3 9,11,t15     | Coakley et al., 2009                |
| Bifidobacterium bifidum       | BHI            | 0.8          | 30     | 72    | 55.6–72%                | C18:3 9,11,t15     | Villar-Tajadura et al., 2014        |
| Bifidobacterium pseudolongum subsp. pseudolongum | Milk | 0.75 mg/mL | 37 | 24 | ND*                     | C18:3 9,11,t15     | Gorissen et al., 2012              |
|                               | BHI            | 0.5         | 37     | 72    | 78.4%                   | C18:3 9,11,t15     | Gorissen et al., 2010               |
|                               | BHI            | 0.5         | 37     | 72    | 62.7%                   | C18:3 9,11,t15     | Gorissen et al., 2010               |

Besides *L. plantarum*, other lactobacilli strains have also been tested (Gorissen et al., 2011): *L. curvatus* LMG 13553, *L. plantarum* ATCC 8014, *L. plantarum* IMDO 130201, *L. plantarum* LMG 6907, *L. plantarum* LMG 13556, *L. plantarum* LMG 17682, *L. sakei* 23K, *L. sakei* C91, *L. sakei* CTC 494, and *L. sakei* LMG 13558. These strains assayed with 0.5 mg/mL of pure LA or α-LNA for 72 h at 37°C (except for *L. sakei* and *L. curvatus* strains that were cultured at 30°C) converted α-LNA to different CLNA isomers (RLA and C18:3 9,11,t15). The authors concluded that α-LNA conversion was strain-dependent, since there were significant statistical differences. On the other hand, LA was converted by only three of these 10 strains, pointing out to higher efficiency in converting α-LNA than LA. The highest CLNA conversion percentage was observed for *L. sakei* LMG 13558 (60.1%). Using this particular bacteria strain in a bioreactor with MRS medium and 0.5 mg/mL of pure LA or α-LNA for 48 h, the authors tested the effects of temperature (20, 25, 30, and 37°C) and pH (5.5 and 6.2) during fermentation. Temperature and pH influenced LA and α-LNA conversion as well as bacterial growth. These authors also reported the presence of linoleate isomerase genes in the genome of strains able to produce CLA and CLNA.

**CLNA production by propionibacteria**

To the best of our knowledge, Verhulst et al. (1987) were the first to evidence CLNA production using propionibacteria strains. The strains were cultured under anaerobic conditions for 48 h at 37°C in Brain Heart Infusion (BHI) medium with 20 µg/mL of different pure substrates, including α-LNA and γ-LNA (C18:3 c6,c9,c12) in order to test their capability in isomerizing polyunsaturated long chain fatty acids. *Propionibacterium freudenreichii* subsp. *freudenreichii* NCIB 8896 and NCIB 5959, *P. freudenreichii* subsp. *shermanii* NCIB 10585, NCIB 5964, and NCIB 8099, *P. acidi-propionic* NCIB 8070 and NCIB 5958, and *P. Tehranicum* NCIB 5965 converted, on total, over 30% of the α-LNA to RLA. *Propionibacterium acnes* were not able to convert α-LNA but did form C18:3 c6,t10,c12 (50.3%) and C18:3 t9,t11,c15 (3.2%). Comparing with the
above-mentioned study, this strain showed higher conversion capacity than other eight bacteria. Nevertheless, it may be noted that *P. freudenreichii* subsp. *shermanii* 9093 was cultured for more time at lower temperatures and in a different medium. This strain was also grown in the presence of a 20-fold higher α-LNA concentration (0.45 vs. 0.02 mg/mL).

In spite of good production of CLNA by propionibacteria strains, this group is less studied among CLNA-producer bacteria. Thus, more studies are needed to support the α-LNA isomerizing ability of these microorganisms.

**CLNA production by bifidobacteria**

According to current bibliography, this genus is the most promising one and has attracted much of the investigations. Coakley et al. (2003) investigated α-LNA isomerization ability of 6 CLA-producing bifidobacteria derived from human intestinal sources: *Bifidobacterium lactis* Bb12 and *Bifidobacterium breve* NCIMB 702258, NCTC 11815, NCIMB 8815, NCIMB 8807, and DPC 6035. The strains were grown in *cys*-MRS medium containing 0.24 and 0.41 mg/mL of pure α-LNA at 37°C for 42 h under anaerobic conditions. The *B. breve* strains were capable of converting α-LNA to RLA and C18:3 9,11,15 at conversion rates of 67.6 to 80.7% (assaying 0.24 mg/mL) and 49.4 to 79.1% (with 0.41 mg/mL). Recently, *Bifidobacterium breve* strains, isolated from the human breast milk, isomerized 94–97% of 0.5 mg/mL α-LNA to RLA in milk-based medium (24 h at 37°C) under anaerobic conditions (Villar-Tajadura et al., 2014).

According to Jiang et al. (1998), the conversion of LA to CLA may be a detoxification mechanism. Previous results suggest that α-LNA is more toxic than LA, since *B. bifidum*, *B. pseudolongum* subsp. *pseudolongum* and *B. breve* strains, cultured at 37°C for 72 h in *cys*-MRS medium with 0.5 mg/mL of α-LNA under anaerobic conditions, showed a higher conversion rate of α-LNA (55.6–78.4%) than LA (19.5–53.5%), and strains growth was inhibited in the presence of α-LNA (Gorissen et al., 2010).

Gorissen et al. (2012) conducted a research work with the aim of producing a fermented milk with enhanced CLA and CLNA concentrations. Microorganisms (*B. bifidum*, *B. breve*, and *B. pseudolongum* subsp. *pseudolongum*) were inoculated in milk containing sunflower (5.00 mg/mL of LA) and rapeseed oils (0.75 mg/mL of α-LNA) at 37°C for 24 h under anaerobic conditions with or without co-inoculation of a commercial yoghurt starter culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* Danisco LYO120 and *Streptococcus thermophilus* YC180). The starter culture was added to increase the amount of free LA and α-LNA due to their lipolytic activity. The results demonstrated that the growth of bifidobacteria strains was not inhibited when the starter culture was added, however, no significant differences were found for rumenic acid content in comparison to control (0.08 mg/g fermented milk), and CLNA was not even detected with or without starter culture. The results suggest that the amount of free LA and α-LNA was too low, possibly because of lack of lipolytic activity.

Furthermore, human-derived bifidobacteria strains previously described as CLA and CLNA producers, *B. breve* DPC 6330 and *B. breve* NCIMB 702258 (Hennessy et al., 2012), have been incorporated in mice diet to evaluate the in vivo production and their effect on lipid metabolism (Wall et al., 2009; Barrett et al., 2012). A significant modification of lipid profiles in the liver, adipose tissue, brain, serum, and intestines was observed, being palmitoleic acid (C16:1 c9), eicosapentaenoic acid (C20:5 ω3), and docosahexaenoic acid (C22:6 ω6), the principal FAs altered. CLA levels were also significantly higher in comparison to the control samples. However, CLA could have been metabolized from CLNA, since this has been observed in vivo (Tsuzuki et al., 2006). These results are very promising but further research must be accomplished since mechanisms behind lipid profile modification are still unknown.

In comparison to other groups of microorganisms, bifidobacteria appears to be the best CLNA producers showing high potential for future elaboration of new CLNA-enriched foods.

**Linoleate isomerase as a screening tool**

Commercial high CLA oils are currently produced mainly through alkaline isomerization of LA. However, this strategy has not been utilized for the elaboration of high CLNA to bring more easily available sources. Furthermore, chemical catalysts are not isomer-selective, resulting in a mixture of different positional isomers that cannot be selected (Reaney et al., 1999). An alternative to this process is the bioconversion of LA and α-LNA into pure CLA and CLNA single isomers using specific enzymes. As mentioned previously, specific microorganisms are capable of producing CLA and CLNA as intermediates in the biological process of biohydrogenation. Isomerization represents the initial step of this pathway, occurring in FA containing a c9,c12 double bond structure. Linoleate isomerase (LAI) (EC 5.2.1.5) is the enzyme responsible for the conversion of LA and α-LNA in their conjugated forms by forming conjugated double bonds from the c9,c12 double bond system (Bauman et al., 1999). The presence of a double bond at the c15 position in α-LNA has little or no effect on the system and therefore biohydrogenation of LNA follows the same pathway as that of LA in rumen microorganisms (Kepler and Tove, 1967).

Linoleate isomerase from the rumen bacterium *B. fibrisolvens* was first described in 1967 (Kepler and Tove, 1967). Thereafter, LAI enzymes from *Clostridium sporogenes* (Peng et al., 2007) and *Propionibacterium acnes* (Liavonchanka et al., 2006) have been characterized. Several attempts to solubilize the *B. fibrisolvens* LAI were ineffective, suggesting an association of this enzyme to the cell membrane (Kepler and Tove, 1967). Similarly, the *C. sporogenes* LAI is membrane-associated and both isomerases are only active on substrates containing c9,c12 double bonds in C18 fatty acids (Peng et al., 2007). However, *C. sporogenes* LAI shows higher *V* max and *K* m with LNA than with LA, suggesting that the extra double bond increases the reaction rate while decreasing its affinity for the substrate. On the other hand, although the *P. acnes* LAI has similar substrate specificity to that of *B. fibrisolvens* and *C. sporogenes*, it is an intracellular soluble cytoplasmic protein capable of converting LA to CLA t10,c12 single isomer (Rosberg-Cody et al., 2007). Due to its high solubility and high catalytic activity, *P.
acnes LAI is a promising candidate for the biosynthesis of conjugated fatty acids (He et al., 2015).

To the present date, different LAI protein sequences have been annotated in GenBank, including sequences of P. acnes, L. acidophilus, L. plantarum, L. reuteri, Lactococcus lactis ssp. lactis, B. dentium, B. breve, Rhodococcus erythropolis, L. delbrueckii ssp. bulgaricus, P. freudenreichii ssp. shermanii, B. fibrisolvens, and C. sporogenes strains. According to the amino acid identity percentage of annotated sequences, LAs can be divided into four groups: (1) LAI from P. acnes that did not show any identity to other LAs; (2) LAI from L. reuteri, L. acidophilus, and Lact. lactis ssp. lactis; (3) LAs of L. plantarum and R. erythropolis, and (4) LAs of B. dentium and B. breve (Farmani et al., 2010). The P. acnes isomerase shares no significant sequence homology to other enzymes except a flavin-binding domain in the N-terminal region (Deng et al., 2007). In addition, with the exception of P. acnes, LAs from the species mentioned have significant homology with myosin cross-reactive antigen (MRCA) proteins. MCRA-like LAs are mostly 9,11-isomerases and are mainly associated with the cell membrane (Farmani et al., 2010).

Molecular techniques have been recently used to screen and study the putative LAI of different species within the genera Lactobacillus and bifidobacteria (Macouzet et al., 2010; Gorissen et al., 2011). Among Lactobacillus strains studied, the LAI gene sequence is identical and the putative LAI gene sequence was only identified in the genome of strains able to produce CLA and CLNA isomers. Therefore, genotypic screening appears to be a reliable method to detect the presence of CLA and CLNA producing strains.

In order to increase CLA and CLNA production levels, the use of recombinant technologies has been tested during the last decade. The LAI gene has been successfully introduced in other microorganisms such as Saccharomyces cerevisiae (Hornung et al., 2005; He et al., 2015), Escherichia coli (Rosberg-Cody et al., 2007; Luo et al., 2013), and Bacillus spp. (Saengkerdsuub, 2013). In addition, LAI gene has been introduced in plants such as tobacco seeds (Hornung et al., 2005) and rice (Kohn-Murase et al., 2006). Most of the studies focused on the recombinant production of LAs from P. acnes, since it is more difficult to develop a recombinant biocatalyst based on membrane-bound enzymes and problems regarding their solubility may rise during recombinant production.

Stability of CLNA in enriched products

A bioactive compound must be stable after elaboration and during storage period of the product. Otherwise it may result in the ingestion of hazardous compounds (e.g. peroxides from lipid oxidation) and/or an insufficient intake as to obtain the effective dose. There is an utter lack of data describing the stability of high CLNA products during storage and after elaboration.

However, interesting information can be obtained from the studies focused on CLA. Its stability in enriched food matrices has been investigated in terms of influence of elaboration (i.e. thermal processing) and storage conditions (time and temperature). Thus, CLA content was stable in naturally enriched milk after UHT processing (142°C; 2 s), since total CLA content (4.67 g/100 g of FA) was similar to that in raw milk (4.68 g/100 g of FA) (Jones et al., 2005). Furthermore, after elaboration of butter and cheese, CLA contents were 4.34 g/100 g of fat and 4.80 g/100 g of fat, respectively, and not significantly different from that in the UHT milk. However, Campbell et al. (2003) observed a significant loss of C18:2 c9,t11 in fortified milk (2% CLA) after high temperature, short time (HTST) pasteurization (77.2°C; 16 s).

The first clear insight about the effects of processing were reported after assaying cooking and frying of milk fat (200–300°C, 15 min), as elaidic acid (C18:1 t9) increased proportionally to temperature as a result of isomerization of oleic acid (OA, C18:1 c9), while C18:1 c9,t11 decreased (Precht et al., 1999). In further experiments, it was reported that linoleic acid can be oxidized at high temperatures to produce CLA isomers (namely C18:2 c9, t11 and C18:2 t10, c12) or isomerize to trans, trans moieties through an intramolecular sigmatropic rearrangement (Destaillats and Angers, 2005) that can also affect CLA isomers (Destaillats et al., 2005). According to these findings, it could be thought that these effects occur only with high temperature processing. However, it was demonstrated that trans fatty acids and CLA isomers in milk increased as a result of pasteurization and sterilization processes in agreement with the above-mentioned reactions (Herzallah et al., 2005; Rodríguez-Alcalá et al., 2014).

It is known that the deodorization of linseed oil lead, as expected, to the formation of trans, trans isomers (Wolff, 1993), while oxidation conditions during derivatization of pomegranate oil transformed PUA into trans, trans compounds (Chen et al., 2007). Moreover, the stability of CLNA to oxidation was compared with thiatof CLA, LA, and α-LNA, and it was quite lower than in those former fatty acids (Yang et al. 2009).

Finally, other investigations focused in the study on the stability of CLA during storage. It was found elsewhere that throughout 14 days at 4°C in dark or light exposure, the fatty acid concentration, including CLA (18:2 c9,t11), did not change in naturally enriched pasteurized milk (Lynch et al., 2007), while CLA butter aged (8 weeks, 6°C) in a similar way compared with regular samples (Mallia et al., 2008). On the other hand, skimmed milk added with high CLA oils showed loss of C18:2 c9, t11, while in fresh cheese loss was for C18:2 c11, t13, C18:2 c9, c11, and C18:2 c10, c12 as a result of microbiological growth after 3 and 10 weeks of refrigerated storage (Campbell et al., 2003; Rodríguez-Alcalá and Fontecha, 2007). Recent results seem to confirm these findings, as in yoghurt elaborated with cow’s milk, the concentration of C18:1 c9, t11 decreased after 14 days (5°C) (Serafeimidou et al., 2013), while in feta cheese, levels decreased during aging (Laskaridis et al., 2013).

Then studies concerning CLA stability in enriched products after temperature treatments have shown that this FA is affected by temperature, occurring changes in both total content and isomers distribution. Due to recent interest in CLNA as well as the lack of rich edible and commercial sources, little is known about the thermal and storage stability of these compounds. These issues must be considered in the future research works; available data suggest that temperature will increase
trans moieties, while during storage if microbiological growth takes place, some CLNA isomers may decrease. However, chemical differences with CLA, as well as presence of other compounds in the assayed high CLNA sources (e.g. antioxidants in oils), may lead to unexpected results.

Conclusions and future challenges

At the current moment, a high number of investigations are focused on the utilization of CLNA isomers as bioactive compounds and their addition to foods. This interest is based on the previous promising results showing anticancer, anti-inflammatory, antioxidant, and anti-obesity properties both in vitro and in vivo at lower doses than with CLA. However, its effectiveness at human level has not been studied deeply enough and therefore future research is needed to know a more accurate dose instance of 0.8–3 g/d that the current bibliography points out.

Another challenge in the utilization of these compounds as a functional ingredient is how to obtain high rich sources. At the current moment, ruminant products are discarded, as natural products have very low concentrations, and feeding strategies are obtaining useful improvements. Vegetable seed oils bring a high variety of concentrations, but until now, only pomegranate seed oil is commercially available from edible sources. Research works studying the microbiological production have reported that strains of bifidobacteria, lactobacilli, and propionibacteria are able to convert LNA into CLNA. However, the production seems to be far from the effective dose and few studies had obtained results in the elaboration of foodstuffs. Nevertheless, it has been elsewhere found that bifidobacteria strains showed in vivo production as well as modification of lipid metabolism in rats toward a healthier fatty acid profile in specific tissues. This brings new interesting possibilities buthow these strains exert these effects is unknown.

Finally, when foods with enhanced contents of CLNA were obtained, it would also be needed to know as how elaboration and storage would affect the fatty acid composition of such products. The current knowledge suggests that thermal treatment can alter the distribution of conjugated isomers, while microbiological growth during storage can decrease their content. These effects may change the recommended intake of commercially high CLNA products.

The main outcome of this review on the state of the art about the possible utilization of CLNA isomers as bioactive ingredients in the development of new functional foods is that although CLNA appears to have a great potential, future research has to focus on the positive health effects on humans, methods to obtain high concentration sources, and the issues regarding stability and safety during elaboration and storage.

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