Chapter 3

Conjugated Linoleic and Linolenic Acid Production by Bacteria: Development of Functional Foods

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Additional information is available at the end of the chapter

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1. Introduction

Over the years, the biological significance of conjugated fatty acids has been demonstrated. Among them, there are two that are present naturally in milk and dairy products, from ruminant origin which have been intensively studied in recent times: conjugated linoleic acid and conjugated linolenic acid.

Conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid (c9,c12-C18:2, LA) with a conjugated double bond. It is a natural compound mainly found in ruminant products such as meat, milk and other dairy food that represent the main source of CLA for humans. Of the two biologically important isomers, c9,t11 is the most prevalent one comprising around 80 to 90% of total CLA in ruminant products, and t10,c12 is present in lower amounts as 3-5% of total CLA [1].

CLA is formed as an intermediate product of the biohydrogenation (BH) process that occurs in rumen, as multi-step mechanism carried out by different microorganisms on unsaturated fatty acids to produce stearic acid (C18:0). Also , it can be produced by desaturation of trans vaccenic acid (t11-C18:1, TVA), process that occurs in different tissues such as mammary gland,.

Conjugated linolenic acid (CLNA) are representing by different conjugated isomers of the linolenic acid (c9, c12, c15-C18:3, LNA). It is also resulting of the ruminal microbial metabolism on fatty acids present in foods, but they are also found in some plant seed oils, like pomegranate seed oil rich in punicic acid (c9,t11, c13-CLNA) [2-3] and tung seed oil where α-eleostearic acid (c9,t11,t13-CLNA) content is about 70% [2-3].

Both conjugated fatty acids has undoubted effects on health, with important biological functions demonstrated in animal models, making them a target of intensive study.

Over the years, CLA has received great attention due to their beneficial properties on health. There exist near 28 different CLA isomers produced by natural and industrial process during fatty acid hydrogenation [4], but the most important according to their biological effects are c9, t11 and t10,c12 forms. However, CLA isomer in milk fat according to importance are c9,t11 (around 80%) followed by t7,c9, which is quantitatively the second most important reaching level so high as 3 to 16 % of total CLA [5]. Factors affecting CLA content in milk, such as the food of ruminants [6-7], the animal breeding type and the stage of lactation [8] were widely reported.

Many studies demonstrated the action of CLA as anti-carcinogenic [9], anti-diabetic [10] and immune-modulator [11] compound. Although there is no agreement regarding its function on fat metabolism, some authors revealed that its consumption also decreases the fat deposition [12].

In addition, CLA produced trough chemical isomerization of LA is offer as dietary supplement in many countries. However, unexpected isomers are produced by this process. To consider CLA as a nutraceutical or medicinal compound, a selective isomer production must be done.

On the other hand, CLNA showed anti-carcinogenesis effects *in vitro* and *in vivo* models [9, 13-14] and other isomers were reported as hypolipidemic compound in human liver derived HepG2 cells [15]. Moreover, it was demonstrated that CLNA exhibits stronger cytotoxic effect on tumoral cells than CLA isomers [16].

Since the most important sources of both conjugated fatty acids for human consumption are milk and dairy products, and due to the microbial production of these compounds, several attempts are being developed to increase its content in food using natural process for it production. In the field of human and animal health, it is interesting to understand the potential beneficial role of selection of bacteria with the ability to form conjugated fatty acids to be then included in foods. Thus, the processed products could be considered as functional foods and sometimes as probiotics, as we detailed below.

2. Ruminal production of conjugated linoleic and linolenic acid

Fatty acids are present in forages and concentrate feeds, mainly as esterified form, mostly present as phospholipids and glycolipids in forrages and triglycerides in plant seeds, comonly used in concentrates.

The two most abundant fatty acids from animal diet are linoleic and linolenic acids. Both are incorporated through diet and once they reach the rumen, are extensively modified by microbial enzymes, such as lipases. These enzymes produce as results LA and LNA as free form for further reactions of isomerization and hydrogenation.

The biohydrogenation of both fatty acids occur in a similar manner, but differ in the intermediate products, as shown in *Figure 1*.

Hydrogenation of linoleic acid produces as first intermediate c9, t11-CLA isomer, by a process where the double bond at carbon-12 position is transferred to carbon-11, carried out by linoleate isomerase (EC 5.3.1.5, LAI). The second step, is the rapid conversion to t11-C18:1 (trans vaccenic acid, TVA) by a reduction mechanism and further hydrogenated to stearic acid (C18:0) [17].

The other CLA isomer resulting of rumen metabolism is t10, c12, which is produced by different microorganism such as *Butyrivibrio fibrisolvens* [18] and *Megasphaera eldsenii* YJ-4 [19]. But the hidrogenation of this isomer not produced TVA but c6, t10-C18:2 which is further converted to C18:0. According to authors, some bacteria can produce hydroxiacids previously to its conversion to CLA isomers [20-21].

As we mentioned above, the other fatty acid of importance in ruminant feeding is linolenic acid, which is also converted to C18:0 by microbial action. In this pathway, LNA is isomerized at *cis*-12 position forming as first intermediate product c9,t11,c15 isomer, named conjugated linolenic acid (CLNA). This compound is further reduced to t11,c15-C18:2 and after that converted to three different products: t11-C18:1; c15-C18:1 and t15-C18:1. As shown in *Figure 1*, only t11-C18:1 is reduced up to C18:0.

Note that metabolic pathway of both LA and LNA fatty acids produce TVA as result of a reduction process, forming conjugated fatty acids as intermediate products. All conjugated fatty acids are absorbed by intestine cells, reason why they are further present in milk and meat fat [22-23].

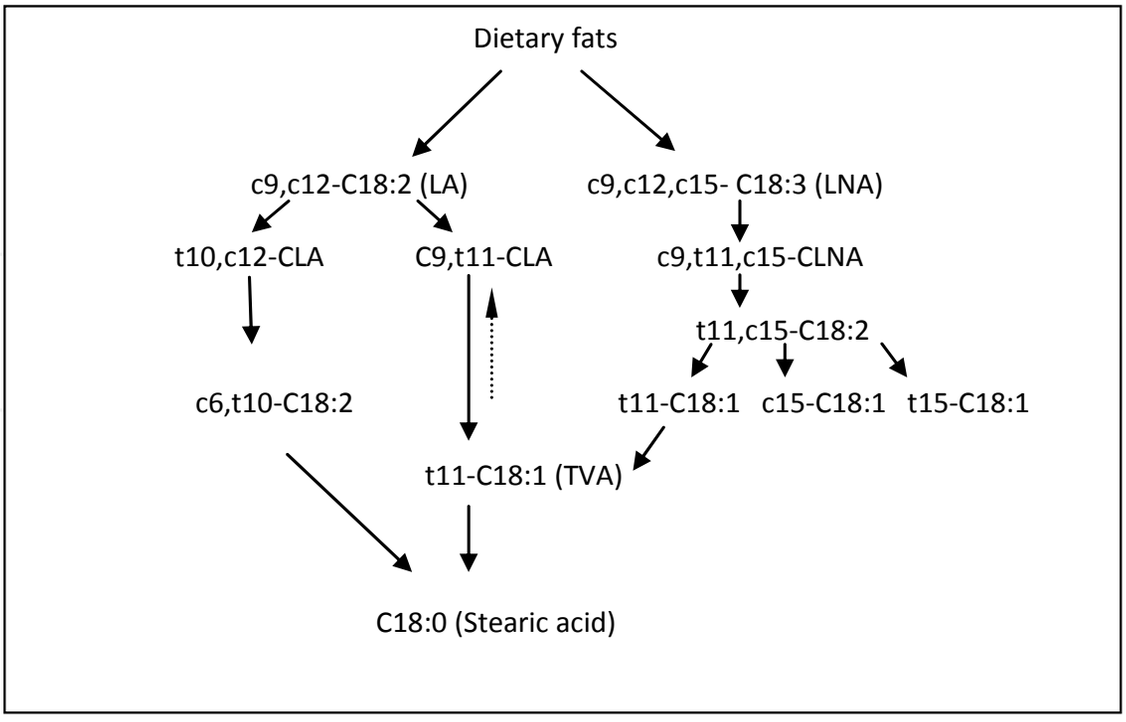

Figure 1. Biohydrogenation process of linoleic and linolenic fatty acid in rumen (adapted from Harfoot and Hazlewood [17]) and endogenous synthesis of CLA in mammary gland (dotted arrow).

Of the rumen microorganism, bacteria are largely responsible for biohydrogenation of unsaturated fatty acids and protozoa seem to be of only minor importance [17].

Kemp and Lander [24] divided bacteria into two groups according to reactions and end products of biohydrogenation. Group A includes those bacteria able to hydrogenate linoleic acid and α-linolenic acid producing t11- C18:1 as an end product. On the other hand, Group B bacteria including those able to use t11-C18:1 as one of the main substrates to produce stearic acid as end product. A listing of the bacteria species of both groups is provided in the review by Harfoot and Hazlewood [17].

Instead of ruminal biohydrogenation, there is another CLA synthesis pathway carried out through the Δ9-desaturase enzyme activity on trans-vaccenic acid (t11-C18:1-TVA) in different tissues, especially in the mammary gland [25]. This endogenous synthesis of CLA is the responsible for most of CLA level found in milk fat, being according to findings around a 64 % [25] to > 80% [26].

But other pathway of CLNA isomers synthesis, in addition to ruminal production, was not yet evidenced. For that reason, its content in ruminant milk apparently comes exclusively from BH of linolenic acid and diet.

As results of microorganism metabolism, many isomers originated in rumen are present in milk. Due to biological properties of both conjugated fatty acids researchers are looking to develop natural foods enriched in CLA and CLNA and thus increase daily intake by humans.

As it was previously mentioned, ruminant milk and meat are the most abundant sources of CLA for humans. Different studies have demonstrated that CLA content of ruminant milk and meat products varies between 4-6 mg/g fat [27-29]. From this value, near the 80 to 90% corresponds to the c9,t11 isomer [30-31]. However, the concentration of CLA can vary widely, where differences are largely related to diet. So, milk fatty acid profile can be modified according to animal feeding.

In the last years, different supplements such as vegetal oils, animal fat, natural pasture and seeds were used to improve fatty acid profile of milk, to attempt higher levels of CLA [32-33] or CLNA [22, 34].

Respect to CLA synthesis in non-ruminant animal, an increase on CLA content in tissues was evidenced in studies using rats [35] and mice [23] after TVA supplementation.

In humans, an endogenous synthesis of CLA was also shown by Adolf *et al*. [36]. But tisular human production in human tissues is so low, that the concentration found in tissues are directly related to food consumption.

Although ruminant foods are the richest source of CLA for humans, it is also found in monogastric animal products, such as swine [37], chicken [30], turkey [30], fish and rabbit [38] meat but in much lower levels. Among south-american camelids, CLA was determined in llama´s (*Lama glama*) milk [39]. Vegetable oils contain little CLA and according to some

authors no CLA content were evidenced in vegetal oils. Typical values of CLA in non-ruminant foods are given in *Table 1*.

Product	Total CLA (mg/ 100 g of fat)	Author
Meat		
Turkey	0.25	Chin *et al*. [30]
Fish	0.01-0.09	Fristche and Steinhart [105]
Swine	0.3-0.9	Ross *et al*.[37]
Rabbit	0.11	Fristche and Steinhart [105]
Chicken	0.09-0.2	Chin *et al*. [30]
Eggs yolk	N.D N.D	Raes *et al*. [106] Gultemiriam *et al.* [107]
Milk		
Human	0.1	Park *et al*. [108]
Horse	0.05-0.12	Jahreis *et al.* [8]
Sow	0.19-0.27	Jahreis *et al*. [8]
Llama *(Lama glama)*	0.7	Schoos *et al.* [39]
Vegetable oils	<0.01	Fristche and Steinhart [105]

ND: not determined

Table 1. CLA content in non-ruminant foods

So as CLA, different CLNA isomers occur naturally, some of which could be formed by ruminal biohydrogenation and further incorporated into milk and meat fat.

Only a few studies were done respect to CLNA content in ruminant products and according to data informed, the only isomer present in cow milk is c9,t11,c15 form [40] while in muscle is also present c9,t13,c15 isomer [40].

CLNA content in milk is around of 0.3-0.39 mg/g fat [23, 40]. At the present, the effect of diet on CLNA concentration in milk was only reported by one work, where cows not fed with extruded linseed (control) have no CLNA in milk, but linseed supplementation in diet increased both CLA and CLNA content, reaching the latest fatty acid a value of 0.15% of total fatty acids [22]. In this study, CLNA was also present only as c9, t11, c15 isomer.

CLNA content in non-ruminant products were determined in different seed oils, being the most abundant source of these fatty acid isomers *(Table 2).* Moreover, tung, pomegranate and catalpa oils showed high level of CLNA but in different isomer ratio. On this way, punicic acid (c9, t11, c15-CLNA) is contained about 72% in pomegranate seed oil [3]. In bitter gourd oil and tung seed oil the main isomer present correspond to α-eleostearic acid (c9,t11,t13-CLNA) in about 60% and 70%, respectively [3, 41]. Catalpa seed oil contains CLNA at a level of 31 %, found as catalpic acid (t9, t11, c12-CLNA) isomer.

Product	CLNA (%)	Author
Pomegranate oil	75 86	Suzuki *et al*. [3] Yücel *et al*. [2]
Catalpa oil	27.5 31	Yücel *et al*.[2] Suzuki *et al.* [3]
Bitter gourd oil	60	Yücel *et al.* [2]; Suzuki *et al.* [3]
Tung oil	70	Suzuki *et al*. [3]
Cow milk	0.3-0.39	Loor *et al*. [23] Plourde *et al*. [40]

Table 2. CLNA content in cow milk and seed oils

3. CLA recommended human intake

CLA concentration in dairy products widely varied according to data reported (0.55–9.12 mg/g fat), but even though are lower than required to achieve a biological effect in humans.

Biological properties after CLA administration is depending on isomer and doses administered and the period of study. Those, studies on animal models reported anti-atherosclerosis effect after 0.1-1% of total CLA per day to rabbits [42]. Moreover, anti-carcinogenic effect was determined by authors using levels from 0.5% to 4% into the diet [43-44].

Although the action mechanism is not well understood, CLA was reported as antioxidant compound in animals and *in vitro* models [15].

Just as there are variations in experimental models about effective doses of CLA, depending on animal model and the biological effect evaluated, the recommended dose from human daily intake also widely varied.

In general, by extrapolation of results found in animals, the recommended CLA daily intake is around 0.35 to 1 g/day [15]. Some authors estimated a daily dose of 650 mg [45], but other studies considered that higher doses (3.0 to 4.2 g/day) are adequate to reduce body fat mass [46-47].

However, at the present the real consumption in different countries is lower than recommended dose. Studies on German population estimated a daily CLA intake of 0.35 to 0.43 g for men and women, respectively [38]. In other countries, CLA daily intake was informed so lower as 120 to 140 mg per day [27].

A few epidemiological studies were done in humans, and evidence show that no all isomers are absorbed to a similar extent. According to result is difficult to predict the impact of CLA consumption on humans and the preventive effect of isomers.

Thus, a short-term (4 to 12 weeks) human studies showed that 2.2 g/d, administered as a mixture of c9,t11 and t10,c12 isomers, produces a decrease on inflammatory markers [48]. A higher dose (3 g/d) were used by Moloney *et al.* [49] who found an increase on HDL levels and a decrease on the ratio of LDL cholesterol to HDL cholesterol, but did not show positive effect on insulin levels in diabetics patients.

Smedman *et al.* [50] reported a reduction of body fat in humans after consumption of 4.2 g/d of a mixture of CLA isomer (c9,t11 and t10,c12) during 12 weeks.

Even though there are many positive findings about CLA supplementation by animals, some negative aspects were informed by other authors, such as the induction of fatty liver and spleen and resistance to insulin [51].

Studies concerning to increase CLA content in foods receives great attention since bacterial inclusion improves CLA levels in some fermented dairy products or could generate CLA at intestinal level after a probiotic administration. In this way, studies on bacterial CLA or CLNA production are relevant in this field, and are detailed in this chapter.

4. Bacterial CLA and CLNA production

The ruminal anaerobe *Butyrivibrio fibrisolvens* was the first bacteria were CLA production was evidenced [18]. After years, it was revealed that not only ruminal bacteria were able to form CLA. So, microorganisms isolated from dairy products, human and animal intestine were demonstrated as CLA-producing bacteria, including lactic acid bacteria (LAB) and bifidobacteria. At the present, *Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus plantarum; Lactobacillus brevis, Lactobacillus acidophilus; Lactococcus lactis, Propionibaterium freudenrehichii, Bifidobacterium sp, Streptococcus*, among others, were able to form CLA [52-54].

Some years ago, it was reported the formation of another isomers of conjugated fatty acid from α and γ-linolenic in *Lactobacillus plantarum*, named as CLNA [21]. Even though this conjugated fatty acid production was reported since 2003, it was only recently informed for bifidobacterium strains [55-56].

Conjugation of linoleic and linolenic acid were proposed as a detoxification mechanism to avoid the growth inhibition effect of fatty acid on bacteria [57-58].

CLA/CLNA production varied among strains being influenced by substrate concentration, culture media, temperature and time of fermentation, among other factors. The isomer formed is also strain-dependent, showing some microorganism the production of only one isomer while others produce two or more CLA/CLNA forms.

As an example of the influence of culture condition, Ogawa et al. (2001)[59] informed CLA production by *L. acidophilus* cultured in microaerophilia conditions, but when bacteria were cultured in aerophilia conditions not CLA formation was determined.

Nowadays, different processes are being carried out to increase CLA production by strains. So, Lin et al (2005) [60] immobilized cells of Lactobacilli strains in two matrix (chitosan and poliacrilamide). In this study, *L. delbruekii* ssp. *bulgaricus* and *L. acidophilus* showed higher CLA production than not immobilized cells. Washed cell instead of growth cultures is another way to produce high CLA levels ([20, 59, 61].

Further studies informed that the uses of enzyme extract of *L. acidophilus* at 50ºC and pH 5 produce more than eight CLA isomers, being around 48% as c,t/t,c form [62].

The transformation of linoleic and linolenic acid to the conjugated form is carried out by linoleate isomerase (LAI) enzyme, which is bound to the bacterial membrane [63]. This enzyme will be treated in other section of this chapter.

At the present, the *in vitro* bioproduction of conjugated fatty acids has been shown in lactic acid bacteria (LAB), propionibacteria and bifidobacteria strains.

A different mechanism of CLA production via 10-OH-C18:1 seems to be the most common pathway in human intestine bacteria according to McIntosh *et al.* (2009) [64], who evidenced this metabolic pathway in *Roseburia*, *Ruminococcus* and other intestinal strains.

5. Lactic acid bacteria (LAB)

CLA production by LAB strains were informed during years. The mechanism, isomer and optimum condition for CLA formation makes these the most variable group on the literature.

Some strains as *L. plantarum* AKU 1009a were informed as CLA-producing bacteria via a two-step reaction: first the hydration of linoleic acid to 10-hydroxy-18:1, followed by dehydration of the resulting hydroxy acid to CLA. In this strain, CLA was formed as c9,t11 (CLA1) and t10,c12 (CLA2) isomers [21].

Xu *et al*. [65] also informed CLA production as c9,t11 and t10,c12 isomer of CLA, at different ratio, in LAB and propionibacteria strains cultured in a fat milk model supplemented with hydrolized soy oil for 24 to 48 h. Among these, *L. acidophilus*, *L. casei*, *L. plantarum*, *E. faecium*, *L. rhamnosus*, *Pediococcus (Ped.) acidilactici* and yogurt cultures (mixture of *L. delbruekii* ssp. *bulgaricus* and *Str. salivarius* ssp. *thermophilus*, 1:1 ratio) were reported as CLA-producing bacteria in the mentioned condition. Increasing time from 24 to 48 h did not increase CLA content, except in *Ped. acidilactici* and *L. rhamnosus* strains. The main isomer found was c9,t11 followed by t10,c12 after 24 h of incubation, except in *E. faecium* were t10,c12 were not determined.

The ability to produce CLA in Lactobacilli strains from human origin was also informed by Lee *et al* [66]. In this study, *L. rhamnosus, L. paracasei* and *L. pentosus* also showed different CLA isomer ratio production. So, *L. rhamnosus* and *L. pentosus* were able to transform LA to c9, t11 and t10,c12- CLA, while *L. paracasei* only produce the c9,t11 isomer.

Other study revealed six LAB able to form CLA after 24 h of incubation, varying percentage of LA conversion between 17% and 36%. Here, *L. casei*, *L. rhamnosus*, and *Strep. thermophilus* showed the highest LA conversion in MRS broth, and increased two- or threefold in milk than MRS broth [53]. *Strep. thermophilus* has importance by it uses as starter culture during fermentation process of dairy products.

Some authors informed a positive correlation between CLA production and tolerance to LA [53, 67] using different substrate concentration. However, the efficiency of CLA production in some LAB and *bifidobacterium* decreases at higher levels of free LA in the medium [53].

Other studies using LAB showed CLA production mainly as c9,t11 form (60-65 %), followed by t10,c12 (30-32%) and other minor isomers like t9,t11 and t10,t12 (2-5%) in *L. acidophilus*, *L. plantarum* and *Lact. lactis* cultured in MRS broth and skim milk during 24 h. [68].

In a recent work, a low CLA production was informed by strains of *L. sakei* and *L. curvatus* (1.6 % and 4.2 %, respectively), commonly present in meat fermentation as starter cultures or natural microorganism [56, 69].

The reaction sequence of isomerization of LA seems to involve different steps according to bacterial strain.

Respect to CLNA production, *L. plantarum* AKU 1009a was able to transform ricinoleic acid to CLA (CLA1 and CLA2) [20]. Further studies demonstrated that this lactobacilli strain has the capacity of use α- and γ-linoleic acids as substrate to generate the corresponding conjugated trienoic acids [21] named CALA and CGLA, respectively. Authors reported a CALA production rate of 40% under two isomer forms: c9, t11, c15-C18:3 (CALA 1, 67% of total CALA) and t9,t11,c15-C18:3 (CALA 2, 33% of total CALA). A higher CGLA production rate was determined in this study (68%) as a mixture of two isomer: c6, c9, t11-C18:3 (CGLA 1, 40 % of total CGLA) and c6, t9, t11-C18:3 (CGLA 2, 60% of total CGLA).

Recently, determination of CLNA production by other LAB strains were informed [69]. Among these, a high production levels were determined in *L. sakei* and *L. curvatus*, reaching a percentage of conversion of 22.4 % and 60.1 %, respectively. Authors evidenced that the isomerization process of LA to CLA and LNA to CLNA is different according to LAB strain, so as isomer resulting after culturing. Some microorganisms were able to form both conjugated fatty acids, but predominantly convert LNA to CLNA, while others not were able to form CLA but effectively converted LNA to CLNA. Results are given in *Table 3*.

Strain	c9,t11	t10,c12	Other isomer	LA conversion (%)	Author
L. curvatus	+	+	-	1.6%	Gorissen *et al*. [69]
L. plantarum	+	+	-	4.6%	Gorissen *et al*. [69]
	+	-	+	N.D	Kishino *et al.* [20]
	+	+	-	N.D	Ogawa *et al.* [109]
	+	+	-	N.D	Rodríguez-alcalá *et al.* [68]
	+	+	-	N.D	Xu *et al.* [65]
L. sakei	+	+	-	4.2	Gorissen *et al.* [69]
L. reuteri	+	+		26	Lee *et al.* [110]
L. rhamnosus	+	+	-		Lee *et al.* [66]
	+	+	-		Ogawa *et al.* [109]
	+	-	-	34	Van Nieuwenhove *et al.* [53]
L. paracasei	+	-	-	N.D	Lee *et al.* [66]
L. pentosus	+	+	-	N.D	Lee *et al.* [66]
	+	+	-		Ogawa *et al.* [109]

Strep. thermophilus	+	-	-	33	Van Nieuwenhove *et al.* [53]
L. brevis	+	+	-	N.D	Ogawa *et al.* [109]
L.curvatus	+	+	-	1.6	Gorissen *et al.* [69]
L. acidophilus	+	-	-	20	Van Nieuwenhove *et al.* [53]
	+	+	-	N.D	Ogawa *et al.* [109]
	+	+	-	N.D	Xu *et al.* [65]
L. reuteri	N.I	N.I	N.I	26	Lee *et al.* [110]
Lact. lactis	+	+	-	N.D	Rodríguez- Alcalá *et al.* [68]

+: positive production. -: no production. N.D: not determined. N.I: not informed

Table 3. CLA production by LAB strains cultured in presence of free LA

6. Propionibacteria

Propionibacteria are commonly present in milk and dairy products and some species play an important role in the creation of cheeses, such as emmental cheese. Propionibacteria represents another important group of bacteria where the capacity of LA isomerization *in vitro* was demonstrated, being relevant since it could be included in fermented products as cheeses. So, *P. freudenrehichii* was able to produce CLA mainly as c9,t11 form according to different studies [58, 65, 70-71] although other author reported eight different isomers of CLA produced by enzyme extract in this bacteria [72].

CLA production in a fat milk model supplemented with hydrolyzed soy oil for 24 to 48 h was demonstrated in two *P. freudenreichii ssp shermanii* and *P. freudenreichii* ssp *freudenreichii* [65]. Higher levels of CLA were determined in skim milk than MRS broth.

The ability of *P. acnes*, isolated from sheep, to form CLA only as t10, c12 form was also evidenced [73].

The results clearly demonstrate that propioniacteria strains show a great variability on CLA production, according to many factors as origin, species, substrate and culture conditions.

To the best of our knowledge, CLNA production by propionibacteria strains was recently evidenced by Henessy *et al.* [74]. In this work, bacteria were culture in presence of different fatty acid used as substrate to evaluate it further conversion into the conjugated form. Thus, LA, α and γ-LNA, stearidonic (c6, c9, c15-C18:4) and other polyunsaturated fatty acids were individually incorporated to culture medium. Strains of *P. freudenreiichii* ssp *shermanii* and *P. freudenreihichii* ssp *freudenreichii* were able to conjugate different PUFA, showing different percentage of conversion of each particular fatty acid. Thus, *P. freudenreiichii* ssp *shermanii* 9093 reached a rate conversion of 50.5; 53.5 and 3.09 for LA, α-LNA and stearidonic acid, respectively. On the other hand, *P. freudenreihichii* ssp *freudenreichii* Propioni-6 reached a conversion rate of 44.65; 8.94; and 3.58 for the same fatty acids. The isomerization process on γ-LNA was not evidenced for these bacteria. The increase of substrate concentration caused a decrease on the percentage of bioconversion (as is shown in *Table 4*).

Strain	c9,t11,c15	t9,t11,c15	CLA (%)	CLNA (%)	Author
L. curvatus LMG 13553	+	+	1.6%	22.4	Gorissen *et al.* [69]
L. plantarum ATCC 8014	+	+	4.6%	26.8	Gorissen *et al.* [69]
L. sakei LMG 13558 CG1	+ +	+ +	4.2 ND	60.1 28.4	Gorissen *et al.* [69]
B. bifidum LMG 10645	+	+	40.7	78.4	Gorissen *et al.* [55]
B. breve LMG 11040	+	+	44	65.5	Gorissen *et al.* [55]
B. breve LMG 11084	+	+	53.5	72.0	Gorissen *et al.* [55]
B. breve LMG 11613	+	+	19.5	55.6	Gorissen *et al.* [55]
B. breve LMG 13194	+	+	24.2	63.3	Gorissen *et al.* [55]
B. pseudolongum ssp pseudolongum LMG 11595	+	+	42.2	62.7	Gorissen *et al.* [55]
B. breve NCIMB 8807*	+	+	66	68	Hennessy *et al.* [74]
B. breve DPC6330*	+	+	67	83	Hennessy *et al.* [74]
B. longum DPC6315*	-	-	12	0.0	Hennessy *et al.* [74]
P. freudenreichii ssp. *freudenreichii* Propioni-6 **	+	+	44.6	8.9	Hennessy *et al.* [74]
P. freudenreichii ssp. *shermanii* 9093**	+	+	50.5	53.5	Hennessy *et al.* [74]

*: production of conjugated isomers of γ-LNA and stearidonic acid were also reported. **: production of conjugated stearidonic acid was also informed. ND: not determined

Table 4. CLNA isomers production by bacteria cultured in presence of α-LNA

7. Bifidobacterium strains

Bifidobacteria are found as normal inhabitants of the human gut and is among the first colonizers of the sterile gastrointestinal tracks of newborns [75]. Due to their health´s benefits on humans, it uses as probiotic strains is indubitable [76]. As results after years of investigations, many functional foods have been developed with the addition of bifidobacteria to the food matrix [77-79].

For this reason, it is not surprising that many studies on the ability of these bacteria to produce CLA have been carried out for a long time.

Bifidobacteria species able to produce CLA was reported at first time by Coakley et al. [57], who informed a considerable interspecies variation. So, *Bifidobacterium breve* and *B. dentium* were the most efficient CLA producers among the range of evaluated strains. The highest percentage of LA conversion was determined for *B. breve,* reaching a value of 65% (c9, t11-CLA). In this study, strains also varied considerably with respect to their tolerance to linoleic acid concentration in the medium.

Other authors showed that strains of *Bifidobacterium breve* and *B. pseudocatenulatum* isolated from human feces, were able to form CLA in a rate conversion of 69% and 78%, respectively [80].

Moreover, CLA production in *B. bifidum* cultured in skim milk, using as substrate hydrolyzed soy oil was reported by Xu *et al*. [65], where authors detected CLA production after 24-48 h only as c9,t11 isomer, and traces of the t10,c12 form.

In a recently study the ability to form CLA in two strains of *B. animalis* were reported [68]. Authors found CLA production from free LA and safflower oil added to MRS broth and skim milk. Strains were able to transform LA to CLA after 24-48 h of incubation. In order to abundance, the most important isomer produced was c9, t11 isomer, followed by t10, c12.

Bifidobacterium breve LMC520 can actively convert linoleic acid to c9,t11-CLA, which is the major isomer derived from microbial conversion according to results from Park *et al.* [81].

The study with the highest number of bifidobacteria were carried out by Gorissen *et al*. [55], which performed a screening of 36 different Bifidobacteria strains to investigate their ability to produce CLA and/or CLNA. As substrate they used free LA and α- LNA, revealing that only six strains were able to convert it to different conjugated fatty acid isomers. Strains were identified as a *Bifidobacterium bifidum*, *Bifidobacterium pseudolongum* and four *B. breve* strains, named *B. breve* LMG 11084, *B. breve* LMG 11613, *B. breve* LMG 13194, *B. bifidum* LMG 10645 and *B. pseudolongum* subsp. *pseudolongum* LMG 11595. Moreover, all strains have been shown to be more efficient in converting LNA to CLNA than LA to CLA, in percentages from 55.6% to 78.4% and 19.5% to 53.5%, respectively. In addition, the CLNA isomers that were mainly found were in order c9, t11, c15-CLNA followed by t9, t11, c15-CLNA isomer.

Hennessy *et al.* [74] also informed about isomerization process of different fatty acids by bifidobacteria strains. Moreover, different PUFA such as stearidonic, araquidonic and docosapentanoic and docosahexanoic acid were supplemented to the culture. A general patron of isomerization was determined on *B. breve* and *B. longum* strains, being able to transform LA, α and γ-LNA and stearidonic acid to it conjugated form. As was observed in propionibacteria, the percentage of conversion varied among strains, showing around 12 to 67% of LA conversion, mainly into c9, t11 and t10,c12 isomer. α- LNA was converted among 0 to 83% among strains, and lower rate conversion was determined for γ-LNA (0.5- 37%). The conjugation of stearidonic acid varied from 3.8 to 27%. *B. breve* DPC6330 was the most effective conjugated fatty acid producer, showing a bioconversion rate of 70% for LA, 90% of α-LNA, 17% for γ-LNA and 28% for stearidonic acid.

As well as different ability to isomerize fatty acids was determined in LAB and propionibacteria, bifidobacteria also exhibit a wide range of bioconversion rate. Many factors affect the mechanism of the fatty acids isomerization, such as culture conditions and substrate concentration. The production of different isomers ratio was reported for all evaluated strains.

To the best of our knowledge, this is the only work reporting the conjugation of stearidonic acid by bacteria. Results are given in *Table 4*.

8. Alternative substrate to CLA production

Although free fatty acids is the most commonly substrate employed by authors to analyze CLA or CLNA production by strains, alternative substrates are being evaluated. Many studies using vegetable oils (hydrolyzed or not hydrolyzed) and mono or dilinoleins as exogenous source of fatty acids were determined to be further incorporated to food matrix. Therefore, bacteria must have the ability to hydrolyze the triglycerides and liberate linoleic acid or linolenic acid for further conversion. Only hydrolyzed oils can offer the fatty acid as free form.

While vegetable oils are the richest source of linoleic and linolenic acid, data about the utilization of monolinolein by *B. breve* were informed. This strain, from human origin, was able to generate CLA at higher bioconversion rate than free LA or dilinolein was added to the medium [82].

CLA production in milk system models was described by many authors using vegetal oils as substrate for further isomerization. At the present, soy, sunflower, canola, castor and safflower oils were used as source of linoleic acid [20, 61, 68, 83].

Kishino *et al*.[20] determined CLA production in *L. plantarum* using castor oil and ricinoleic acid as substrate, showing the same end product than using free LA. Moreover, the production of the previously reported hydroxyacids as intermediate compounds, were also evidenced in the assay.

CLA formation by LAB and *Bifidobacterium* strain using safflower oil as LA source added to skim milk at 1 mg/ml was reported by other authors [68], where they informed that some bacteria produced higher CLA using safflower oil than free linoleic acid in skim milk broth after 24 h of incubation. Among these group of microorganism was *B. animalis*, *L. acidophilus* and *Lact. lactis*.

Among bacteria isolated from rumen, *L. brevis* was reported as CLA-producing strain in presence of sunflower oil [84].

However, there was informed no CLA production after the addition of soy oil to skim milk in *P. freudenreihchii; L. casei*; *L. acidophillus*, *L. plantarum*; *P. acidilactici*, *B. bifidum*, *L. rhamnosus* and *E. faecium* [65]. But once hydrolyzed soy oil was supplemented to the medium as substrate, CLA production from 0.6 to 2.2 mg/g fat was determined in all selected strains.

According to results, the utilization of vegetable oils by bacteria as source of fatty acid for it further isomerization is also depending on metabolism of strains.

9. LAI enzyme

As we previously mentioned, linoleate isomerase is an enzyme present in some bacteria, which is bound to the membrane. In the most of bacteria, CLA production is primarily located in the extracellular phase [71] but it can be also found in the cellular membrane as an structural lipid [80]. Moreover, both LA and CLA incorporated to the membrane represent less than 1.7% of the total amount of CLA formed [80].

CLNA isomers are also found primarily in the cell-free supernatants compared to the cell pellet [55]. Authors reported that around 7% of LNA and 5% of CLNA corresponding to the cellular pellet in bacteria cultured in presence of LNA.

For this reason, the analysis methods of fatty acids of cultures did not involve the remotion of bacterial cells. Moreover, total fatty acids content is necessary to determine the complete bioavailability of those compounds once bacteria are included in a food matrix or is considered as probiotic strain.

At the present, LAI has been isolated from bacteria such as *L. delbrueckii* subsp. *bulgaricus*, *But. fibrisolvens*, *L. acidophilus* and *P. freudenreichii* subsp. s*hermanii* showing some differences.

LAI from *But. fibrisolvens* A-38 was isolated by Park *et al [85]*, determining the molecular weight and partial amino acid sequence of the enzyme. According to findings, this LAI consist of a single polypeptide with a molecular weight of 19 kDa.

Other authors isolated and characterized the LAI from *L. reuteri* MRS8 [86], showing a molecular weight of more than 100 kDa. In this study, the optimal activity of the enzyme was in the pH range of 4.7 to 5.4.

A genotypic identification of LAI gene from ten strains able to form CLA and/or CLNA was recently performed [69]. This work presented the homologies of LAI sequence in a dendrogram comparing to other LAI sequence from known LAB.

Moreover, the molecular weight forms LAI from *L. reuteri*, *P. acnes* and *C. sporogenes* were 68 kDa, 45 kDa and 55 kDa, respectively [87-88].

10. Functional foods and probiotics

The development of healthier food is looking for taking in account their benefits for humans. Among these, dairy products represent a good alternative to manufacture functional and/or probiotic foods. Functional food includes processed food or foods fortified with health-promoting additives. By other hand, probiotics are live microorganisms which when administered in adequate amounts confer a health benefit to the host. Several bacteria are informed as probiotic strains during years, where several positive effects on health have been supported [89].

At the present, conjugated fatty acids have attracted considerable attention because of their potentially beneficial biologic effects. Important properties were attributed to CLA and CLNA, and scientific evidence has been demonstrated both in humans and animal models, including anti-tumor, anti-obese, anti-atherogenic and anti-diabetic activities.

Microbiota present in intestine plays an important physiological rol to the host, modulating some metabolic functions, conferring resistance to microorganism infection and increasing immune response, among other functions.

The bioconversion of LA to CLA and LNA to CLNA by bacteria at intestinal level, result a novel and interesting topic to be developed with the objective to obtain probiotic foods with microorganism able to produce it or functional foods with high levels of CLA and /or CLNA.

The uses of CLA or CLNA-producing bacteria as probiotics have received great attention for nutrition, since many studies evidenced their benefits for the promotion of human health.

It has been demonstrated that isomer of CLA has different function and according to reports t10, c12 is more potent than c9,t11 CLA to prevent cancer cell proliferation [90]. This isomer is also associated to a decrease on body fat in animals [91-92] and humans [93-94].

Previous studies informed that CLA content in cheeses varied according to strain used as starter or adjunct culture [95] and to the ripening time [96]. Therefore, the inclusion of bacteria able to form it during the fermentation process has been received great attention by researchers.

At the present, different functional foods (yogurt, cheese, fermented milk) were manufactured with CLA-producing bacteria, obtaining a final product with a high CLA content. cheeses manufactured with CLA-producing bacteria were developed using sunflower oil as exogenous source of LA, reporting a modification of fatty acids profile in mice tissues after it administration [83]. Mice fed functional cheeses showed a protective effect on viability of intestinal cells after a treatment of 1,2-dimethylhydrazine drug, used as oxidant compound.

Nowadays, CLA production by probiotic bacteria has received special interest in the research field, being well established that bacteria isolated from intestine or fecal samples can form it. However, *in vitro* production was intensely informed, while a few studies have established an *in vivo* CLA production after ingestion of bacteria. Authors revealed that according to administered strain, a high t10, c12 isomer [66, 98] or c9,t11 isomer [99] content in animal tissues occurs.

Linoleic acid excretion in humans is estimate at 340 mg/day [100], being this fatty acid available to further isomerization process by intestinal microbiota. Nevertheless, this local CLA production was only reported after probiotic treatment, but if CLA amount produced is enough to exert a preventive effect require better understanding.

Strains daily administered as probiotic, in a short-term study, produced an increase on CLA systemic content [66]. Authors showed that consumption of *L. rhamnosus* PL60 (10^7-10^9 CFU/day) during 8 weeks increased t10, c12 isomer content in plasma and tissues of diet-induced obese mice. Animals receiving PL60 showed a significant reduction of fat adipose tissue (epididymal and perineal). No liver steatosis were observed in this study, being the most adverse effect informed to t10, c12-CLA. The increasing amount of CLA in tissues after oral treatment with *L. rhamnosus* was explained as an intestinal production once bacterium has been colonized the intestine. Lower leptin levels in PL60 group were also informed. Obese mice selection as animal model was supported by t10, c12-CLA as the main isomer formed by this probiotic strain.

Another work supporting the generation of CLA at intestine using animal models have also reported by same researchers and in this study, they use as probiotic strain *L. plantarum* PL62 in obese mice, at daily dose of 10^7-10^9 CFU/mice. The presence of PL62 was determined in fecal samples after the first week of its intake, and after 5 weeks of feeding a weight reduction in mice receiving PL62 was determined. Similar results were observed after two experimental doses. Respect to CLA, as in the previous study, the main isomer formed by bacterium was t10,c12-CLA [98].

So, both human bacteria *L. rhamnosus* PL60 and *L. plantarum* PL62 were demonstrated to be able to form *in vivo* CLA [66, 98].

But. fibrisolvens from goat rumen was able to rapidly convert LA to CLA and LNA to CLNA, showing similar rate conversion for both fatty acids [101]. In this work, selective strain was administered to mice using a daily dose of 10^{11} CFU/mouse, during 4 weeks. After the trial period, a higher CLA amount in feces was determined. CLA content in tissues was also increased after probiotic treatment. Although a high dose of bacteria was employed, no adverse effect was determined. The aim of this study was to develop a probiotic for animals to generate a continuous CLA production and absorption.

The administration of a mix of bacteria able to form CLA as c9, t11 and t10, c12, called VSL3 was used as probiotic for mice administration [102]. The combination of all strains (*L. casei, L. plantarum, L. acidophilus, L. delbrueckii subsp bulgaricus, B. infantis, B. breve, B. longum, Strep. salivarius subsp. thermophilus*) did not increase CLA production compared with individual strains. Probiotic was prepared as lyophilized form and mice were fed 30 μL of probiotic (0.03 g VSL3 in 10 ml water) for 3 days. Feces were collected at day 0 and 3, and were incubated with LA. Results shown that murine feces with LA after administering VSL3 yielded 100-fold more CLA than feces collected prior to VSL3 feeding. This work also reported that the incorporation of probiotic into conditioned medium produced a reduction of viability and induced apoptosis of HT- 29 and Caco-2 cells.

Another important work using bacteria able to produce CLA as probiotics for animal models was showed by Wall et al. [99]. The administration of *B. breve* NCIMB 702258 to mice and pigs, combined with dietary linoleic acid, showed changes on fatty acid composition of liver and adipose tissues. Higher levels of c9, t11 in liver tissues were determined for both animals receiving *B. breve*, and were also associated with reductions of the pro-inflammatory cytokines level.

Recently, an study to investigate if recombinant lactobacillus expressing LAI (from *P. acnes*, producing t10, c12 isomer) administered to mice produce changes on fatty acids profile was carried out [103]. Authors found that after a daily administration of *L. paracasei* NFBC 338 (10^9 UFC/mouse) during 8 weeks, and 4-fold increase of t10, c12 content in adipose tissue was produced comparing with control mice group. Moreover, in liver a 2.5-fold higher level of the same isomer was reported in treatment group. To the best of our knowledge, this is the only work about using genetically modified strains with the ability to produce t10, c12-CLA, administered as probiotic in mice.

There is few data respect to probiotic administration and *in vivo* CLA production in humans. Lee and Lee [104] reported the effect of PL60 consumption by humans. Here, volunteers consumed PL60 as freeze-dried at a dose of 1g/day (10^{12} CFU/g) during 3 weeks. After one week of uptake, PL60 was recovered from feces, as was previously determined in mice. Respect to CLA content in tissues, both c9,t11 and t10,c12 isomers were higher respect to day 0 of treatment (baseline). Leptin levels were also lower at the end of the study.

11. Conclusion

Although one of the most effective method to increase CLA uptake by humans consist of increase CLA levels in milk and dairy products by modification of animal diet or the inclusion of bacteria able to form it during manufacture process, in the last years the *in vivo* CLA production appears as an alternative way to make it.

Since CLA was recognized as an important biolipid with health benefic properties, there was an increasing interest on this field. However, there is another conjugated fatty acid recently included in studies: conjugated linolenic acid (CLNA). This fatty acid is also generating great attention since anti-atherogenic properties were attributed to them. Some bacteria could produce CLNA using as substrate linolenic acid. CLNA isomers in foods and its biological effects in animal models were lesser understanding than CLA, being the mechanism of it production by bacteria recently investigated. So, in the literature there is not yet recommended dose for this compound for humans.

Development of functional foods enriched on conjugated fatty acids is being extensively studied by researchers, since benefits of health properties were related to humans. The physiological role of conjugated fatty acids like CLA or CLNA is well documented on the literature.

The ability of some species of lactic acid bacteria, propionibacteria and bifidobacteria to *in vitro* conjugate the LA and/or LNA has been established over the years. Manufacture of functional food enriched in conjugated fatty acids by using it as starter or adjunct culture is a promising topic to be developed.

The variation on CLA and CLNA production among bacteria depends on many factors such as intrinsic characteristic of each particular strain, conditions of experimental design and methodology for isomer determination, among others. For this reason, studies must be carefully done before the inclusion of strain during food manufacture.

Few authors have demonstrated the action of bacteria intake on *in vivo* CLA production using experimental animal models and human, but results are promising in this field.

Instead of some technological developments have been performed, many points remain undiscovered at this issue. Some aspects of technological processed foods must be considered, such as CLA-enriched products are also high in fat, being difficult to recommend a single daily dose of CLA after food intake. As we earlier mentioned, not all isomers are incorporated at the same way into tissues fat.

Respect to microorganisms able to form conjugated fatty acids, it is not unreasonable to assume a production of these bioactive compounds at intestinal level, since fatty acid substrate are present in human diet.

Further studies are necessary to understand the kinetic mechanism of it particular production. Questions such as if LAI enzyme is the responsible for both LA and LNA conversion need to be clarified so as the factors determining the isomer production by each strain.

Indeed, taking in account the lack of information respect to some epidemiological and technological aspects of conjugated fatty acids, further studies are required to fully understand the utility of CLA and CLNA in disease prevention. The development of products as probiotic or functional foods to ensure the bioavailability of both compounds for humans is a valuable strategy to be considered.

List of abbreviations

But.: *Butyrivibrio*
B.: *Bifidobacterium*
C.: *Clostridium*
c: cis
CALA: conjugated of alpha linolenic acid
CFU: colony forming unit
CGLA: conjugated of gamma linolenic acid
CLA: Conjugated linoleic acid
CLNA: Conjugated linolenic acid
E.: Enterococcus
L.: *Lactobacillus*
LA: linoleic acid (c9,c12-C18:2)
LAB: lactic acid bacteria
Lac.: *Lactococcus*
LAI: linoleate isomerase
LNA: linolenic acid (c9,c12,c15-C18:3)
P.: Propionibacteria
Ped.: Pediococcus
Strep.: *Streptococcus*
t: trans
TVA: trans vaccenic acid (t11-C18:1)

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